

## THESIS / THÈSE

### DOCTOR OF SCIENCES

#### Soil carbon sequestration in heathlands the effects of climate change on fungi

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# **Voorwoord**

## Voorwoord

Deze doctoraatsthesis had nooit verwezenlijkt kunnen worden zonder de steun van een heleboel mensen. In eerste instantie wil ik mijn beide promotoren, Francois en Frederik, hartelijk bedanken voor mij bij te staan op verschillende vlakken doorheen dit traject. Jullie hebben beiden een aantal unieke positieve eigenschappen op zowel professioneel als persoonlijk vlak die ervoor zorgden dat ik altijd met veel plezier met jullie heb samengewerkt. Ook wil ik de andere leden van mijn commissie, Natalie, Richard en Fons, hartelijk bedanken voor al de hulp en het vertrouwen gedurende de vier jaar van mijn doctoraatsonderzoek. Anderen die mijn onderzoek mee richting hebben gegeven door regelmatig feedback te geven, zoals oa Jan, Joske, Laura, Natascha en Bram, verdienen daarvoor ook alle lof. Ook wil ik de nog niet vernoemde leden van mijn jury, Erik en Sofie, bedanken voor hun input. Tot slot zou mijn doctoraatsthesis nooit tot stand zijn gekomen zonder de hulp van de studenten die ik heb begeleid: Talha, Mathias, Sherilyn, Ester, Deniz en Jente, allen hartelijk bedankt voor jullie hulp!

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# Summary

## Summary

Natural ecosystems store large quantities of carbon in their soils, thereby preventing it from ending up in the atmosphere and contribute to climate change. A key question is whether climate change increases or decreases the capacity of soils to sequester carbon, and hence whether ecosystems will buffer or accelerate climate change. However, experimentally *in situ* observed changes in soil carbon contents under climate change simulations are very variable and the underlying mechanisms are poorly understood. Heathlands are rare, semi-natural ecosystems with soils dominated by fungi and relatively high carbon sequestration rates. These systems might thus play an important role in our understanding of the effects of climate change on soil carbon sequestration. Therefore, in this PhD, we investigate how heathland soil fungi are affected by climate change, as changes in soil fungal functioning to a large extent drive the observed changes in heathland soil carbon sequestration.

In **Chapter 1**, the difficulty to parameterize a simple mechanistic food web model that simulates the effect of climate change on soil carbon sequestration indicated that we currently lack basic empirical data on species interactions and stress tolerances. Therefore, we focused in the thesis on the stress ecology of and interactions between fungi, as they are the most important group of organisms with respect to carbon sequestration in heathland soils. But in order to expose heathland soil fungi to abiotic stressors in laboratory experiments, we had to isolate as many fungal species as possible. Therefore, in **Chapter 2**, we tested four methods and seven growth media for their efficiency in isolating soil fungi. All four tested isolation methods, that have largely varying methodologies, showed high taxon specificity and complementarity. Contrary to expectations, the nutrient composition of the growth medium did not affect cultivation. However, long incubation times did prove to be useful for the isolation of additional fungal taxa. Hence, by using various isolation methods combined with long incubation times, we were able to cultivate a relatively diverse soil fungal community.

In **Chapter 3**, we *in vitro* quantified the tolerance to temperature and water stress (drought) of the isolated fungal taxa by assessing their growth under different treatments. Additionally, we measured several functional traits, such as

melanin content, that are considered to be important direct mechanistic drivers of their tolerance to these abiotic stressors. We found a large variability in stress sensitivities among taxa, whereby fungi were in general tolerant to the applied mild temperature and water stress, but sensitive to high temperature stress. These heathland soil fungi are thus relatively well-adapted to harsh abiotic conditions. Contrary to expectations, the measured functional traits did not explain the variation in abiotic stress tolerance among taxa, which is thus probably driven by other traits than those that we quantified. In **Chapter 4**, we investigated how these abiotic stressors affect the capacity of fungi to grow in presence of a more abundant competitor, which we defined as biotic stress tolerance. We found that fungal growth rates were positively affected by biotic stress under benign conditions, but that interactions between fungi become negative under high warming stress, opposite to the stress gradient hypothesis (SGH). Tolerance to biotic stress was not driven by tolerance to abiotic stress nor intrinsic growth rate of the fungus, at any level of abiotic stress.

These results suggest that global change could potentially impact fungal communities in unpredictable ways. Several perspectives would validate and further complement the gathered knowledge, by addressing how the observed changes in fungal growth rates under biotic and abiotic stress propagate into more complex set-ups and more complex communities and eventually translate into changes in soil carbon sequestration.



# **Samenvatting**



## Samenvatting

Natuurlijke ecosystemen slaan grote hoeveelheden koolstof op in hun bodem, waardoor deze koolstof niet in de atmosfeer terecht komt waar het klimaatverandering veroorzaakt. Een belangrijke vraag is of klimaatverandering de capaciteit van bodems om koolstof vast te leggen verhoogt of verlaagt, en dus of ecosystemen klimaatverandering respectievelijk zullen bufferen of versnellen. Experimenteel in het veld waargenomen veranderingen in koolstofgehaltes in de bodem onder simulaties van klimaatverandering zijn echter zeer variabel en de onderliggende mechanismen hiervoor zijn onduidelijk. Heide is een zeldzaam, half-natuurlijk ecosysteem met bodems die worden gedomineerd door schimmels en relatief hoge koolstofgehaltes. Deze systemen kunnen dus een belangrijke rol spelen om de effecten van klimaatverandering op koolstofvastlegging in bodems beter te begrijpen. Daarom onderzoeken we in dit doctoraat hoe schimmels van heidebodems worden beïnvloed door klimaatverandering, omdat veranderingen in de werking van de schimmels in grote mate de veranderingen in koolstofvastlegging in de bodem bepaald.

In **hoofdstuk 1** gebruikten we een voedselwebmodel dat de effecten van klimaatverandering op koolstofvastlegging in de bodem voorspelt. De moeilijkheid om een eenvoudig voorspelend voedselwebmodel te parameteriseren duidt op het gebrek aan gegevens over interacties tussen soorten en de stresstoleranties van soorten. Daarom hebben we ons in het proefschrift gericht op de stress-sensitiviteit van en de interacties tussen schimmels, omdat ze de belangrijkste groep organismen zijn met betrekking tot koolstofvastlegging in heidegebieden.

Maar om bodemschimmels in laboratoriumexperimenten bloot te stellen aan abiotische stressoren, moesten we zoveel mogelijk schimmelsoorten isoleren. Daarom hebben we in **hoofdstuk 2** vier methoden en zeven voedingssamenstellingen getest op hun efficiëntie bij het isoleren van bodemschimmels uit heidegebied. Alle vier geteste isolatiemethoden vertoonden een hoge specificiteit en complementariteit met betrekking tot de geïsoleerde soorten. In tegenstelling tot de verwachtingen had de voedingssamenstelling van het groeimedium geen invloed op de isolatie. Lange incubatietijden bleken echter wel nuttig te zijn voor het isoleren van extra schimmelsoorten. Met

behulp van verschillende isolatiemethoden in combinatie met lange incubatietijden konden we daarom een relatief diverse gemeenschap van bodemschimmels isoleren en vervolgens gebruiken voor experimenten.

In **hoofdstuk 3** hebben we de tolerantie voor temperatuur- en droogtestress van de geïsoleerde schimmelsoorten gekwantificeerd door hun groei te meten onder verschillende behandelingen. Bovendien hebben we verschillende belangrijke eigenschappen gemeten die worden beschouwd als belangrijke parameters die hun tolerantie voor deze abiotische stressoren bepalen, zoals het melaninegehalte. We vonden een grote variabiliteit in stress-gevoeligheden tussen soorten, waarbij schimmels in het algemeen tolerant waren voor mild verhoogde temperatuur en droogte, maar gevoelig voor een sterk verhoogde temperatuur. Deze heide-bodemschimmels zijn dus relatief goed aangepast aan deze veranderende abiotische omstandigheden. In tegenstelling tot de verwachtingen, verklaarden de gemeten functionele eigenschappen niet de variatie tussen soorten in hun tolerantie voor de abiotische stressoren. De sensitiviteit van schimmels voor deze abiotische stressoren gerelateerd aan klimaatverandering worden dus waarschijnlijk bepaald door andere eigenschappen dan die we hebben gemeten, zoals osmolieten.

In **hoofdstuk 4** hebben we onderzocht hoe de klimaatverandering-gerelateerde abiotische stressoren het vermogen van schimmels om te groeien in aanwezigheid van een andere soort beïnvloedt. We vonden dat de groeisnelheid van schimmels positief werd beïnvloed door de aanwezigheid van een andere soort onder normale omstandigheden (positieve inetracties), maar dat interacties tussen schimmels negatief worden onder verhoogde temperaturen. Dit is volledig omgekeerd aan wat we verwachtten op basis van de stress-gradient hypothese. De veranderende effecten van de aanwezigheid van een andere soort op de groeisnelheden van schimmels onder verschillende condities was niet afhankelijk van hun tolerantie voor de abiotische stressoren noch intrinsieke groeisnelheid van de schimmel.

Deze resultaten suggereren dat klimaatverandering schimmelgemeenschappen mogelijk kan beïnvloeden op onvoorspelbare manieren. Verschillende perspectieven zouden de verzamelde kennis valideren en aanvullen, door verder

## Samenvatting

te onderzoeken hoe de waargenomen veranderingen in groeisnelheden van schimmels onder biotische en abiotische stress zich voortzetten in complexere experimenten en complexere gemeenschappen en zich uiteindelijk vertalen in veranderingen in koolstofvastlegging in de bodem.

# **Table of contents**

## Table of contents

Voorwoord .....	i
Summary .....	iii
Samenvatting .....	vii
Table of contents .....	xi
General Introduction .....	3
Climate change and the mitigating potential of soil carbon sequestration.....	2
Heathland soils to sequester carbon.....	8
The heathland soil food web .....	10
Objectives .....	16
Chapter 1 .....	3
Abstract .....	20
Introduction .....	21
Uncertainty regarding food web structure and function.....	23
Uncertainty regarding the effects of climate change on soil food webs.....	26
Modelling drought stress effects on carbon sequestration .....	28
Results.....	31
Conclusions .....	36
Supplementary Information .....	37
Chapter 2 .....	21
Abstract .....	42
Introduction .....	43
Materials and Methods .....	45
Results.....	49
Discussion .....	54
Conclusions .....	58

Supplementary Information .....	59
Chapter 3 .....	43
Abstract .....	64
Introduction .....	65
Materials and Methods .....	68
Results.....	72
Discussion .....	75
Conclusions .....	80
Supplementary Information .....	81
Chapter 4 .....	65
Abstract .....	86
Introduction .....	87
Materials and Methods .....	89
Results.....	92
Discussion .....	96
Conclusions .....	100
Supplementary Information .....	101
General Discussion.....	103
Science to mitigate climate change: investigating heathland soil fungal functioning .....	104
The heathland soil fungal community is generally well-adapted to temperature and water stress, but fungal tolerance is unpredictable from their traits ....	107
Positive pairwise interactions under benign conditions become negative under temperature stress, are not shaped by abiotic stress tolerance nor intrinsic growth rates.....	109

## Table of contents

Potential consequences of the observed changes in fungal functioning for carbon sequestration .....	111
Perspectives in order to increase the predictability of the effects of climate change on heathland soil community functioning and carbon sequestration	112
Conclusions .....	115
References.....	117
Appendix .....	137
Links between heathland fungal biomass mineralization, melanization and hydrophobicity .....	138

# **General Introduction**



## **Climate change and the mitigating potential of soil carbon sequestration**

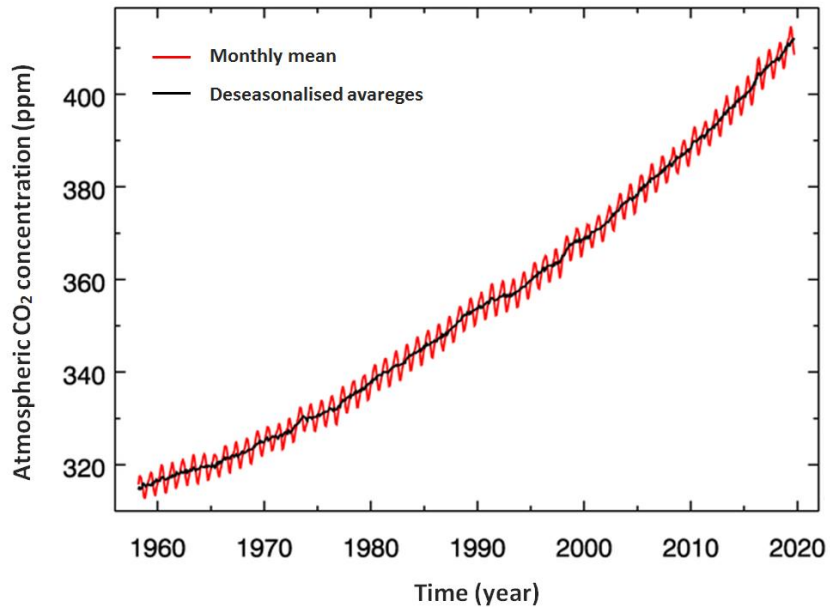
In the biogeochemical carbon cycle, carbon circulates among different reservoirs, thereby undergoing changes of chemical form. Atmospheric carbon dioxide (CO<sub>2</sub>) is transferred to the biotic pool of the terrestrial system and the oceans through photosynthesis and released back into the atmosphere by respiration of micro-organisms through decomposition. The concentration of CO<sub>2</sub> in the atmosphere has increased from approximately 277 parts per million (ppm) in 1750 [1], the beginning of the Industrial Era, to 402.8 ppm in 2016 [2] (Fig. 1), thereby unbalancing the global carbon cycle and inducing climate change due to its radiative forcing effect [3]. The initial atmospheric CO<sub>2</sub> increase above preindustrial levels, around 1750, was primarily caused by land-use change such as deforestation [4] (Fig. 2). From around 1920, emissions from fossil fuels and industry became the dominant source of anthropogenic emissions to the atmosphere, and their relative share has continued to increase until present (Fig. 2). In the last decade (2007-2016), it grew at a rate of, on average, 1.8% per year, slowing down to 0.4% increase per year during 2014-2016.

There are two complementary approaches to deal with this global problem: i) reducing CO<sub>2</sub> emissions and ii) 'capturing' some of the atmospheric CO<sub>2</sub> in pools other than the atmosphere, such as terrestrial ecosystems. The latter is a crucial ecosystem service called carbon sequestration. It is a long-term process which takes advantage of plant photosynthesis, in which atmospheric CO<sub>2</sub> is converted into biomass. Upon mortality, this plant biomass ends up in soils as long-lived soil organic matter (SOM) and is then progressively returned as CO<sub>2</sub> in the atmosphere during its decomposition by soil organisms. Soils contain approximately 2500 Pg (10<sup>15</sup>g) carbon, which is approximately four and three times higher than respectively the biotic and atmospheric pool [5]–[7]. Pools that are large and have low flux rates, such as soils, may regulate long-term trends by sequestering carbon, keeping it away from the atmosphere, where it causes climate change. Due to this carbon sequestration potential, soils have the

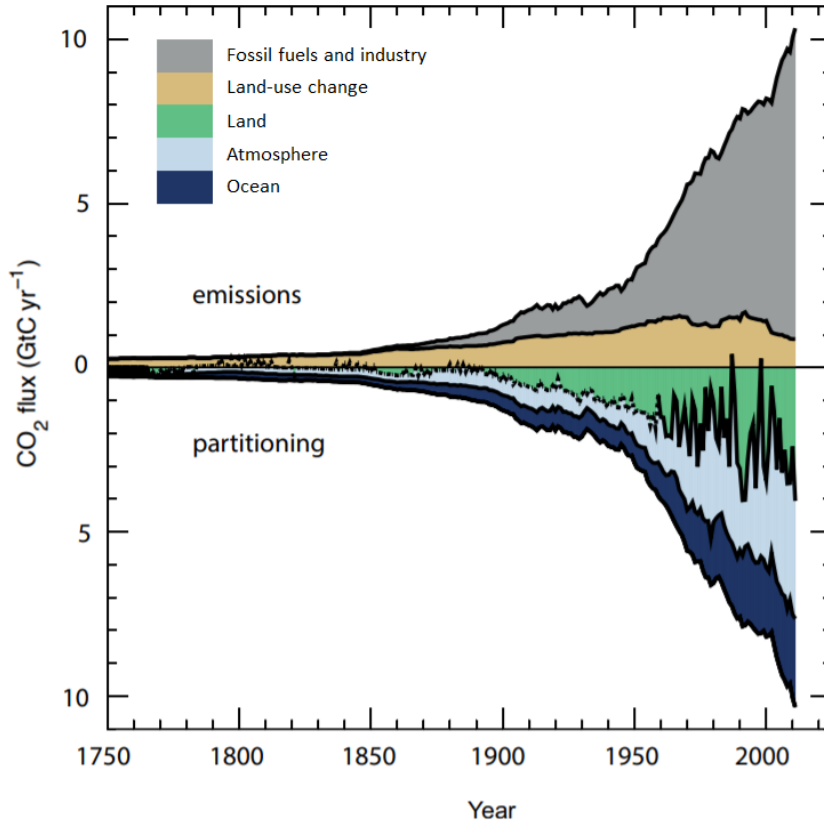
ability to mitigate climate change by safeguarding carbon stores, thereby counteracting the effects of increasing greenhouse gas emissions [7].

During the past decades, terrestrial ecosystems and oceans have been absorbing 'excessive' anthropogenic CO<sub>2</sub> emissions, partially into the soil carbon pool [6], [8], [9]. However, the increase of fossil fuels and industry emissions was only partly compensated by this increased carbon sequestration by land and oceans (Fig. 2), nevertheless leading to the significant increase in atmospheric CO<sub>2</sub> (Fig. 1) that causes climate change. More specifically, during the last decade (2007-2016), 88% of the total emissions were from fossil fuels and industry, and 12% from land-use change (Fig. 3). The total emissions were partly partitioned into oceans (22%) and land (28%), but mainly to the atmosphere (44%), with a remaining unattributed budget imbalance of 5% (Fig. 3). Hence, a key question is whether the capacity of soils to sequester carbon will increase or decrease under further climate change. Evidence is mounting that climate change related extremes such as droughts and warming can lead to a decrease in regional ecosystem carbon stocks [10], thereby creating a positive feedback loop that further strengthens climate change [11].

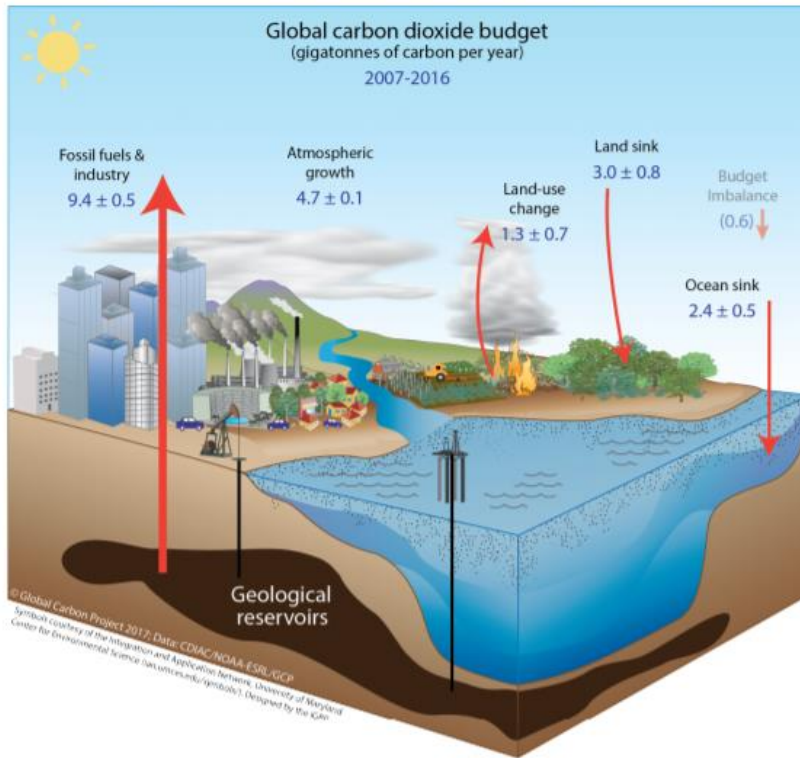
The projected future effects of climate change on terrestrial ecosystems vary regionally. In Europe, the two most important factors are i) increasing average temperatures including more extreme heat waves and ii) altered precipitation regimes with long periods of drought compensated by short-term heavy rainfalls [3] (Fig. 4). Hence, climate change creates multi-stress conditions. Therefore, we focus on the effects of both temperature increases and drought.



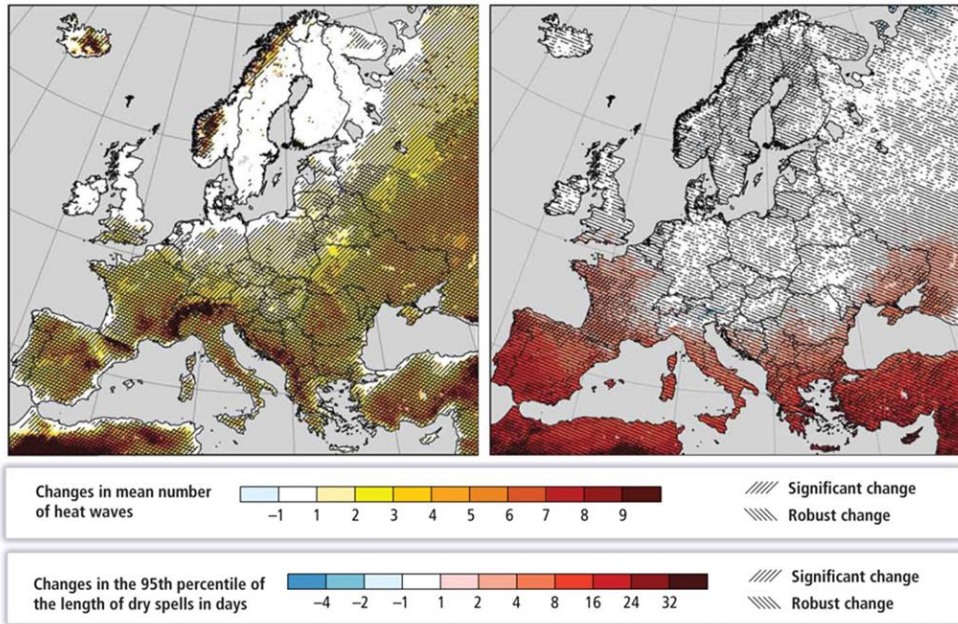
**Figure 1:** Increasing average surface atmospheric CO<sub>2</sub> concentration (in ppm), measured at Mauna Loa Observatory in Hawaii, from Dlugokencky and Tans 2019 [12]. Monthly average, depicting seasonal fluctuations, are depicted in red, deseasonalised averages are in black.



**Figure 2:** Combined components of the global carbon budget as a function of time since the industrial revolution, for emissions from fossil fuels and industry (grey) and land-use change (brown) as well as partitioning in oceans (dark blue), land (green) and the atmosphere (light blue), from Ciais *et al.* 2013 [4]. There is a vast increase in all components apart from land-use change emissions, expressed in gigatonnes of carbon per year.



**Figure 3:** Schematic representation of the overall perturbation of the global carbon cycle caused by anthropogenic activities, averaged globally for the decade 2007-2016, expressed in gigatonnes of carbon per year, illustrating the excessive fossil fuels and industry emissions that cannot be completely partitioned in the land and ocean sink, leading to a vast atmospheric growth, from le Quéré *et al.* 2018 [2].



**Figure 4: Left:** Projected changes in the mean number of heat waves occurring in the months May to September for the period 2071–2100 compared to 1971–2000 (per total of 30 years). Heat waves are defined as periods of more than 5 consecutive days with daily maximum temperature exceeding the mean maximum temperature of the control period (1971–2000) by at least 5°C. **Right:** Projected changes in the 95th percentile of the length of dry spells for the period 2071–2100 compared to 1971–2000 (in days). Dry spells are defined as periods of at least 5 consecutive days with daily precipitation below 1 mm. Hatched areas indicate regions with robust (at least 66% of models agree in the sign of change) and/or statistically significant change (significant on a 95% confidence level using Mann–Whitney U test). Projections are based on the RCP8.5 scenario, from the 2014 IPCC report [3].

## Heathland soils to sequester carbon

European heathlands are semi-natural habitats that developed on acidic sandy soils following forest clearances which started about 4000 years ago [13]. Heathlands currently occur through the Atlantic region of western Europe, from northern Spain, where it has affinities with Mediterranean shrublands to the north-west of Norway, where it has affinities with tundra [14], [15]. Its occurrence is marked by its temperate climate with cool and moist summers and warm winters. At the research area in the National Park Hoge Kempen in Belgium (see further), the mean temperature at 10cm depth was 17°C during spring and summer of 2019 [16], whereby the sharpest increase and decrease over all seasons was approximately 5°C in 5 days. Lowland heathland depends on the occurrence of nutritionally poor and acidic soils called podzols. The plant community composition is characterized by dwarf shrub vegetation of the Ericaceous family, and is especially dominated by *Calluna vulgaris*. Traditional activities that had been responsible for the maintenance of heathland, by preventing regeneration of the forest, include grazing, cutting turf, burning, cutting vegetation for fuel and harvesting the vegetation for fodder [15]. The persistence of heathland soils currently still relies on these management practices. Lowland heathland in Europe extended over a million hectares, but today, mainly due to land-use changes [17], a little over 350 000 remain [18], which represents approximately 1% of total European land area. In Belgium, there has been a reduction from 163 000 to 13 000 hectares since the 19<sup>th</sup> century. Although it currently is protected, losses continue through a combination of a lack of proper management and nitrogen deposition. This causes transition to forests via a grassland stage, with *Molinia caerulea* and *Deschampsia flexuosa* being the main grass species involved in this vegetation succession, leading to a decrease in the amount of soil carbon being sequestered.

Among ecosystems, there is a wide variation in fluxes of carbon between pools. For carbon sequestration in soils, a key factor determining its capacity is the recalcitrance of the plant biomass. Depending on its chemical composition,

different types of plant biomass are decomposed at different rates. Hence, partly due to differences in plant species composition, various ecosystem soils do not trap carbon for the same period of time. Ecosystems bearing plants with a high proportion of recalcitrant compounds therefore have a higher potential for soil carbon sequestration. In contrast to grasses, heathland dwarf-shrub plants such as *Calluna vulgaris* produce high proportions of recalcitrant polyphenolic compounds such as lignin, and are therefore characterized by very slow carbon decomposition rates [14]. Also, contrary to other systems (e.g. coniferous woodlands) where carbon is more or less equally stored in the soil and vegetation, almost the complete carbon fraction of heathlands is found in the soil [7], [19]. The soil carbon stock in dwarf shrub heathlands is on average 88t C/ha [7], which is among the highest of all European biomes, after wetlands and boreal forests. Heathland soils can therefore be considered as ecosystems with a high potential to act as a carbon sink, implying a climate change mitigation potential [6]. Despite the limited occurrence of European heathlands in particular, habitats dominated by Ericoid-vegetation in general are considered to hold approximately 20% of the earth's terrestrial carbon [14]. Also, by applying correct management practices, degraded marginal arable fields can be converted into heathlands [20], thereby increasing the global soil carbon sink. Heathlands thus are semi-natural, cultural landscapes with an important regulating ecosystem service [21] called soil carbon sequestration [22]. But, due to former land use changes and current climate change and difficulties in management, these ecosystems are under major threat.



## The heathland soil food web

The rate of the cycling of carbon in soils is driven by soil organisms through the process of decomposition. The soil microbial community produces extracellular enzymes that degrade the variety of organic substrates into small monomeric compounds which are metabolized and released as CO<sub>2</sub> by respiration, thereby returning the photosynthesized carbon back to the atmosphere. Soil fauna consume other organisms including microbes and also mix and cut plant litter thereby making it better accessible for the enzymes produced by micro-organisms. Hence, the composition and functioning of the soil community controls the decomposition rate of soil organic matter.

### Soil organic matter formation and decomposition

Two major sources of soil organic matter (SOM) input are plant litter and root deposits. The latter mainly consists of soluble sugars. Plant litter composition of *Calluna vulgaris*, that dominates heathlands, consists of approximately 40% cellulose, 30% hemi-celluloses (e.g. pectin) and a relatively high portion of lignin (20-30%). Additionally, microbial necromass and residues have been recognized as a major pathway to SOM formation as it can account for up to 80% of the organic carbon in soils [16]. The relative contribution of these different input sources into the pool of SOM is unknown. However, especially in heathlands, the input of microbial origin is expected to be relatively high because of the high abundance of recalcitrant melanin-rich fungi [23].

In general, there is a negative relationship between decomposition rates of organic matter and its chemical complexity. However, this is heavily influenced by abiotic (e.g. accessibility and stabilization) and biotic (e.g. decomposer enzymatic 'toolbox') factors [24], [25]. Most of the stable carbon in heathland soils is found in deep layers, whereby microbial activity is mainly restricted to the surface soil layer (upper 20 cm) [14]. Therefore, spatial inaccessibility and stabilization are expected to have less influence [26], [27], which advocates the role of substrate recalcitrance and functional community composition in regulating soil organic matter decomposition rates.

### **Ericoid mycorrhizal and saprotrophic fungi are the main decomposers**

Heathlands are characterized by harsh edaphic conditions consisting of very low pH (3-5) and nutrient availabilities. These two abiotic factors imply severe physiological constraints for biota to thrive in these soils. Soil organisms can be divided in primary and secondary consumers, depending on their trophic level. Within heathland soils, microbes are the main primary consumers or decomposers and the microbial decomposition of SOM in heathlands is believed to be mainly driven by fungi, as the contribution of bacteria in acidic soils generally is very minor [28]. Indeed, Haugwitz *et al.* [29] found fungi to be more abundant than bacteria in heathland soils in Denmark, especially under climate change, indicating that fungi generally are more tolerant to climate-change induced stressors than bacteria. Additionally, fungi generally are more capable of degrading complex organic matter than bacteria, whereby they have a more prominent role in the sequestration of soil carbon. Therefore, in this PhD, we focus on the functioning of soil fungi, although it is important to conceive that this is a major simplification, as bacterial groups such as Acidobacteria likely do play an important role in heathland soil carbon sequestration.

The fungal decomposers of heathlands consist of both saprotrophic, free-living fungi and ericoid mycorrhizal fungi (ERM), living in a symbiotic relationship with the Ericaceous plants dominating heathlands, such as *Calluna vulgaris*. Well known ERM taxa that generally are abundant in heathland type soils are *Hymenoscyphus ericae* and *Oidiodendron maius*, saprotrophic fungi include *Penicillium*, *Mortierella*, *Mucor* and *Absidia* [14], [30]. Recent culture-independent molecular methods however revealed a higher diversity of ERM than those typically isolated [31], [32]. Mycorrhizal fungi generally are considered to be obligate symbionts, entirely depending on their host plant for deriving their carbon and consequently for survival. However, *Hymenoscyphus ericae* has been shown to be able to produce enzymes involved in the degradation of (hemi-)celluloses and even polyphenols such as lignin [33]. Additionally, a recent genome study revealed that both *Hymenoscyphus ericae* and especially *Oidiodendron maius* contain genes encoding for a large array of degradative secreted enzymes, often richer and more varied than that of soil saprotrophs, including genes for polysaccharide-degrading enzymes, lipases,

## General Introduction

proteases and enzymes involved in secondary metabolism [34]. Interestingly, there is a closer relationship between ERM and saprotrophs than to those of ectomycorrhizal symbionts (ECM), for which a facultative saprotrophic lifestyle is questioned [35]. Thus, the ERM gene repertoire reveals a capacity for a saprotrophic lifestyle, which may reflect an incomplete transition from a saprotrophic to a mycorrhizal lifestyle, or a versatile life strategy.

The influence of ERM on carbon sequestration depends on the balance between several phenomena [36]. On the one hand, they decrease carbon sequestration by decomposing (highly recalcitrant) organic matter [14], and by doing so they also release other nutrients, which can prime the growth of other soil microorganisms [37] and hence contribute to faster carbon cycling. On the other hand, ERM fungi obtain carbon from their plant partner, and therefore take up more nitrogen per unit of carbon than other soil microorganisms. That makes organic matter harder to decompose, because there are then less nutrients available per unit of carbon, which contributes to slower carbon cycling [38]. Moreover, the biomass of most ERM species is strongly melanized, which makes it harder to degrade [23], and hence contributes to sequester carbon too, this time in fungal biomass, as previously mentioned. Overall, data suggest that the net balance of ERM on carbon sequestration is positive (the effect of C/N ratio and melanization outweighs direct carbon mineralization with associated priming) [14], [39], [40]. Other fungi are saprophytic; they need to forage for their own carbon in the organic matter and are generally less melanized, and are therefore expected to contribute to carbon decomposition.

According to Read and Perez-Moreno [33], there is an overwhelming predominance of the ERM functional group over saprotrophs in heathland ecosystems. However, strong conclusive evidence is lacking due to absence of high-throughput sequencing studies that characterize the fungal community composition of heathland type soils. The importance of research regarding the interactions between these different types of fungi (mycorrhizal vs. saprotrophic) has been stressed for ectomycorrhizal fungi in forests [41], [42], as well as arbuscular mycorrhiza in grasslands [36], but less so for ERM [36]. Read *et al.* (2004) [14] emphasized the need for the evaluation of the relative

contributions of symbiotic and saprotrophic fungi to the processes of carbon storage and cycling in heathlands, particularly in the context of global climate change. In conclusion, we know relatively well the general relative contribution to carbon sequestration of both fungal guilds but knowledge on their abundance and species specific composition and interactive functioning is very scarce.

### **Enchytraeids are the main consumers**

Several groups of heathland soil fauna contribute to the decomposition of SOM, including microbial necromass, thereby contributing to the cycling of carbon. Most abundant fauna groups include collembola (springtails), acari (mites), nematodes and enchytraeid worms [43], [44]. The latter are in terms of biomass the most abundant consumers in nutrient poor acidic organic soils, including heathlands [18]. *Cognettia sphagnetorum* (actually a complex of several cryptic species [19]) is the keystone enchytraeid species with a dominance of up to 95% [45]–[47]. The necromass of enchytraeids and other soil animals is considered to be easily degradable [48], thereby additionally priming SOM decomposition due to an increased microbial activity and nutrient availability [49]. However, excrement of soil fauna can be more recalcitrant than ingested compounds, thereby potentially fostering carbon sequestration. Although knowledge on food preferences of enchytraeid worms has been relatively well studied and synthesized [50], it still remains uncertain whether enchytraeids, and *C. sphagnetorum* in particular, in situ actively forage for fungal mycelium or bulk feed on SOM, as earthworms do in forests. Consequently, their functional role regarding carbon cycling within the heathland soil food web remains uncertain. In conclusion, despite a lot of uncertainty on different contributions, enchytraeids and other soil fauna are assumed to generally accelerate the recycling of the carbon locked in fungal or plant biomass and necromass, hence they reduce carbon sequestration.

### **The effects of climate change**

As carbon sequestration is a long-term process, it will be influenced by climate change. The relationship between climate change and carbon sequestration is thus a reciprocal interaction, whereby sequestration can not only mitigate, but

## General Introduction

also be affected by climate change. Despite considerable research, it remains uncertain whether climate change increases or decreases the capacity of global soils to sequester carbon, and hence whether soils will buffer or further accelerate climate change [11], [51]. Also for heathlands, no consensus has been found on the faith of soil carbon stocks under future climate change, with a large variability in observed long-term effects among field experiments [52]–[54]. The underlying mechanisms of these changes are very poorly understood. A crucial question therefore is whether and how climate change affects the composition and functioning of soil organisms and how this translates into changes in carbon sequestration. The projected future effects of climate change on terrestrial ecosystems vary regionally. In Europe, the two most important factors are i) increasing average temperatures including more extreme heat waves and ii) altered precipitation regimes with long periods of drought compensated by short-term heavy rainfalls [3] (Fig. 4). Hence, climate change creates multi-stress conditions. Therefore, we focus on the effects of both temperature increases and drought. As previously indicated, heathlands will be most prone to temperature and drought stress. Within this thesis we synchronically use the terms drought and water stress, whereby drought (stress) as an environmental change factor induces osmotic water stress in fungi.

Drought stress has been shown to reduce both root exudation [55] and litter production [45], thereby decreasing corresponding carbon substrate inputs. In fungi, drought thus causes osmotic stress [56] and lowers growth rates [57]. It affects soil microbial community structure [29], [58], [59] and decreases overall (enzymatic) activity [59], [60], thereby potentially increasing sequestration of soil carbon. Since melanin is considered an important trait for stress tolerance [61], climate change potentially also selects for highly melanized fungal taxa (potentially more ERM), thereby increasing even more sequestration of carbon. Additionally, fungi are better stress resistant than bacteria, which may lead to an even more pronounced dominance of fungi [62], which could decrease carbon sequestration, since fungi are generally able to degrade more complex organic molecules than bacteria. In enchytraeids, drought causes osmotic stress, increases mortality, decreases survival and impedes reproduction [63], [64].

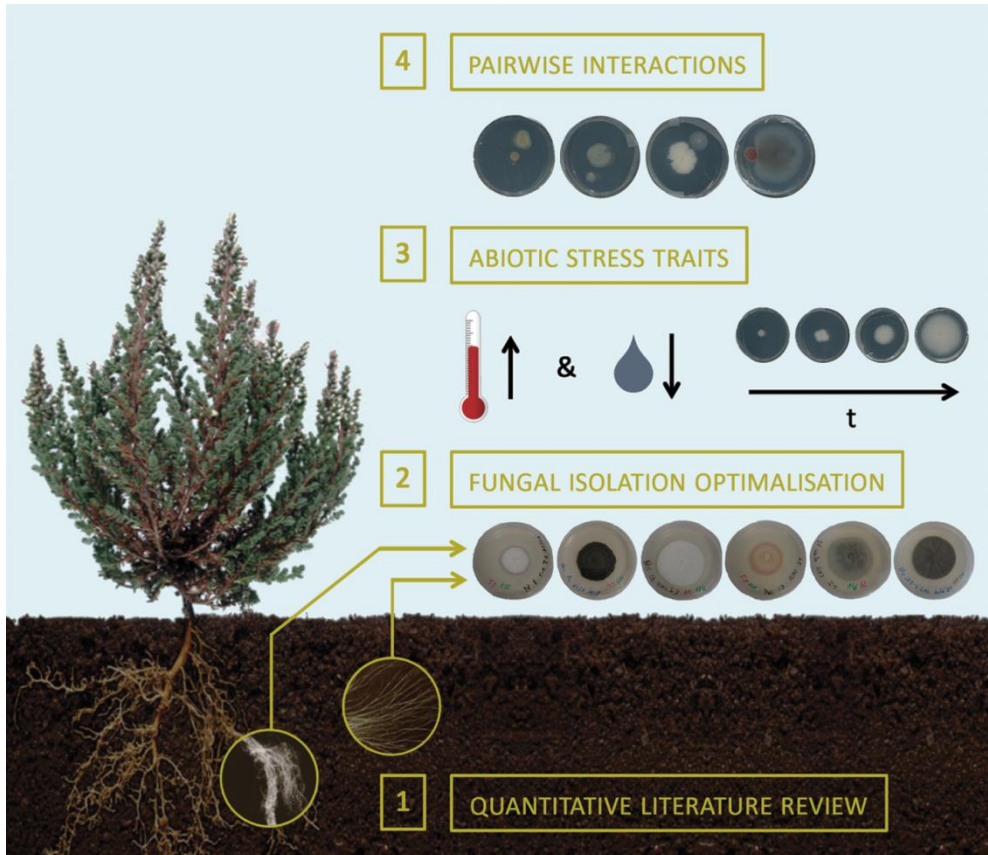
Repeated periodic summer drought stress initially reduces abundance and diversity, with recovery in abundance (but not diversity) after rewetting [45]. Holmstrup *et al.* [44] recently found that warming and drought had low impact on diversity of soil fauna in a temperate heathland. In conclusion, there are some general *in situ* insights on how climate change related stressors broadly affect the functioning of heathland soil organisms and/or changes soil carbon sequestration. However, *in vitro* assessments of the mechanistic drivers of such changes, such as species specific sensitivities and the functional traits that account therefore are severely lacking. Additionally, it is unknown how varying species specific sensitivities affect their interactions and eventually translates into shifts in community composition and consequently soil carbon sequestration.

## Objectives

Hence, research on heathland soil carbon sequestration under climate change thus far mainly focused on broad observational experiments *in situ*, which investigated how soil carbon contents, or proxies therefore, respond to various climate change related stressors. But based on the above, it is clear that carbon sequestration is a complex soil process that is regulated by a network of interactions of communities of soil organisms. Indeed, such *in situ* observations of climate change induced heathland soil carbon content changes are very variable, whereby the mechanistic drivers of these changes at the species to community level are largely unknown. In summary, although dependent on spatiotemporally varying environmental conditions, we know relatively well how each “guild” (ERM, saprotrophs and enchytraeids) theoretically contributes to carbon sequestration: ERM are expected to improve it under certain conditions, while saprotrophic fungi and enchytraeids should have the opposite effect. But experimental data regarding the species-specific community assembly and stress tolerances, as well as on how interactions within and between the communities of these guilds influence their functioning, are thus severely lacking. Furthermore, it is not clear how these processes are affected by climate change.

This lack of basic empirical knowledge is a large obstacle to mechanistically understand the consequences of climate change for heathland soil carbon sequestration. Therefore, the goal of this PhD thesis is to **i)** investigate which uncertain factors on heathland soil food web functioning under climate change are most critical to unravel in order to enhance understanding and predictive capacity (**chapter 1**), **ii)** test which isolation methods are most efficient to cultivate as many heathland soil fungi species as possible for further use in laboratory experiments (**chapter 2**), **iii)** investigate which functional traits shape the sensitivity of these fungi to warming and drought (water stress) (**chapter 3**), and finally **iv)** test how these abiotic stressors affect interactions between fungi (**chapter 4**) (Fig. 5). Thereby, this thesis provides essential basic knowledge for a better understanding of how heathland soil fungi and their interactions are affected by climate change. This is an important first step before

upscaling towards more elaborated and complex food web experiments that could eventually validate *in situ* observed soil carbon sequestration changes.



**Figure 5:** Overview of the four different chapters of the thesis. In chapter 1, we review the information available in the literature regarding soil food web functioning in heathlands and how this is affected by climate change. Additionally, using a simple mathematical model, we quantify which of the identified knowledge gaps are most important to unravel in order to enhance predictive capacity. In chapter 2, in order to use as many of the local heathland soil fungi as possible in laboratory experiments, we use several isolation methods and growth media to increase soil fungal isolation efficiency. Next, we test which functional traits shape the tolerance of these fungi to temperature and water stress (Chapter 3). Finally, we investigate how pairwise interactions between fungi are affected by these abiotic stressors (Chapter 4).





# Chapter 1

## **Food web uncertainties influence predictions of climate change effects on soil carbon sequestration in heathlands**

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*This chapter refers to a related publication, which is added as an appendix at the  
end of the thesis*

WR and FDL designed the experiment in consultation with all other authors. The model was parameterized and simulations analysed by WR, FR, JWS, OF, MPB and FDL. WR wrote the main manuscript text, which was reviewed by all other authors.

## **Abstract**

Carbon cycling models consider soil carbon sequestration a key process for climate change mitigation. However, these models mostly focus on abiotic soil processes and, despite its recognized critical mechanistic role, do not explicitly include interacting soil organisms. Here, we use a literature study to show that even a relatively simple soil community (heathland soils) contains large uncertainties in temporal and spatial food web structure. Next, we used a Lotka-Volterra-based food web model to demonstrate that, due to these uncertainties, climate change can either increase or decrease soil carbon sequestration to varying extents. Both the strength and direction of changes strongly depend on: (1) the main consumer's (enchytraeid worms) feeding preferences; and (2) whether decomposers (fungi) or enchytraeid worms are more sensitive to stress. Hence, even for a soil community with a few dominant functional groups and a simulation model with a few parameters, filling these knowledge gaps is a critical first step towards the explicit integration of soil food web dynamics into carbon cycling models in order to better assess the role soils play in climate change mitigation.

## Introduction

Human-induced climate change affects global carbon cycles and threatens important ecosystem services. Sequestration of carbon into soils as organic matter is considered as an important process of the global carbon cycle because it mitigates climate change by reducing excessive atmospheric CO<sub>2</sub> concentrations [6]. However, a key question is whether climate change increases or decreases the capacity of soils to sequester carbon, and hence whether ecosystems will buffer or accelerate climate change.

Numerous studies, some of which based on predictive simulation models, have projected changes in the soil carbon balance of ecosystems due to various climate change-induced stressors [51], [65], [66]. However, most of these models do not explicitly consider the key role of soil decomposer biota in nutrient and carbon cycling but simulate decomposition through, for example, first-order kinetics that are only affected by abiotic conditions, such as temperature and moisture [65], [66]. In these models, the role of the soil community for biogeochemical cycling is thus not explicitly evaluated.

This strongly contrasts with findings that soil organisms drive the process of organic matter decomposition. The importance of their composition in regulating the effects of climate change on ecosystem processes such as carbon cycling has been discussed extensively [67], [68]. Hence, in contrast to what is implicitly assumed in conventional soil carbon models, shifts in soil community composition due to environmental stressors can have significant consequences for carbon cycling because of associated shifts in ecosystem functioning. Several studies have acknowledged the link between soil food web composition and carbon sequestration, and the need to incorporate this relationship into predictive carbon cycling models [69]–[74]. However, it remains unclear to what extent climate change will affect soil carbon budgets of ecosystems.

In this study, we performed a literature search to identify uncertainties regarding soil food web structure and its consequence for carbon cycling, and the sensitivity of soil biota to environmental stressors. Next, we use a generalized Lotka-Volterra model to investigate how these uncertainties

translate to projections of climate change-induced shifts of soil carbon sequestration. We focus on heathlands because: (1) they are among the most carbon rich soils compared to most other terrestrial systems (Panel 1); and (2) they are relatively simple, which makes modelling them more tractable.

**Panel 1: Heathlands as a study system**

Dry heathlands are semi-natural habitats dominated by ericaceous dwarf-shrubs, primarily the heather species *Calluna vulgaris*, and are a globally relevant study system because they share many similarities with other ericoid dominated shrubland systems, such as tundra [14]. Heathland currently covers an estimated 350 000 ha in Europe, which represents approximately 1% of total land area. Moreover, soil carbon content in heathland is among the highest of all biomes, after wetlands and boreal forests, and can therefore be considered as potentially significant carbon sinks. This ecosystem is under threat from land-use and climate change, which lead to a 10- to 20-fold decline in its occurrence since the middle of the nineteenth century. Available carbon cycling simulation models are less accurate for carbon rich soils, such as heathland, than those for others, such as grasslands [204]. Field experiments further suggest that the effect of climate change related stressors such as drought on heathland soil carbon balances varies considerably among sites, with a tendency of increased sequestration (sink) at drier sites and decreased sequestration (source) at wetter sites [52], [53]. However, the underlying mechanisms of these changes are very poorly understood.

## Uncertainty regarding food web structure and function

Sources of soil organic matter (SOM) input consist of plant litter, root exudates and microbial and soil faunal necromass (Fig. 1). The organic compounds entering the soil have different turnover rates. Solubles are generally less recalcitrant than polysaccharides, which are in turn less recalcitrant than polyphenols. However, their degradability is heavily influenced by abiotic (e.g. accessibility, temperature, moisture) and biotic (e.g. decomposer catabolic 'toolbox') factors [24], [75]. Within heathland soils, microbes are the main decomposers and the microbial decomposition of SOM is mainly driven by fungi, as bacterial abundance is low due to high soil acidity [28]. Two important fungal functional groups are ericoid mycorrhizal fungi and saprotrophic fungi.

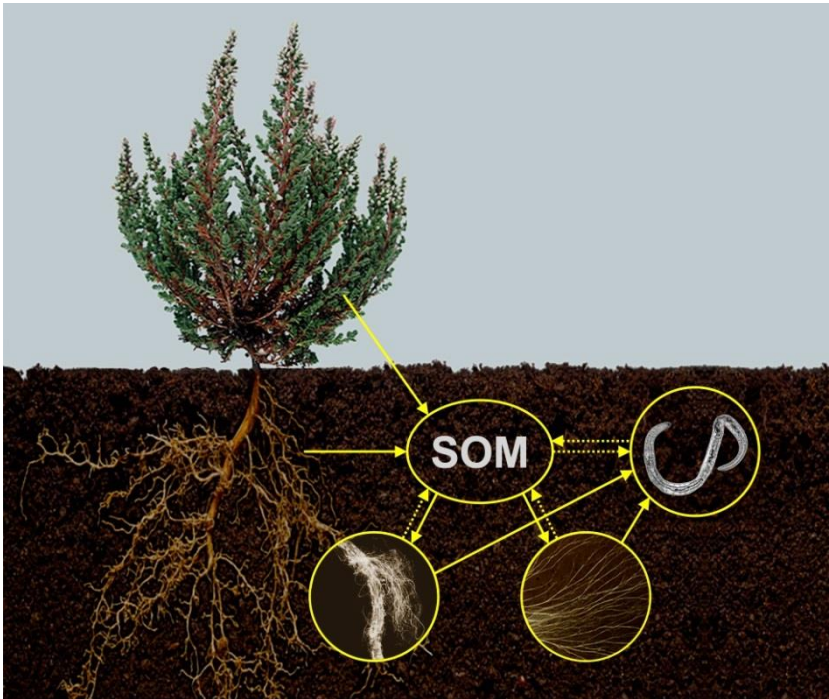
The net effect of fungi on soil carbon sequestration depends on the balance between their effects on carbon loss via decomposition and stabilization of soil organic carbon (SOC) via conversion of assimilated solubles and polysaccharides into more recalcitrant polyphenolic compounds in their fungal tissues, which enter the SOM pool upon mortality. Although microbial necromass varies considerably across ecosystems and is affected by environmental stressors such as drought, it can account for up to 80% of the organic carbon in soil [76]. The contribution of microbial necromass to the soil carbon pool is likely to be high in heathland soils because of the high abundance of recalcitrant melanin-rich fungi [77]. In a side study, we investigated the importance of two important fungal morphological properties, hydrophobicity and melanin content, regarding their contribution for the decomposability of heathland soil fungal necromass (Appendix).

Some groups of soil fauna can contribute to the decomposition of the microbial necromass, such as Collembola (springtails), Acari (mites) and enchytraeid worms. Enchytraeid worms are, in terms of biomass, the most abundant consumers in nutrient poor acidic organic soils [63], including dry heathlands, where *Cognettia sphagnetorum* (actually a complex of several cryptic species [78]) is the keystone species with an estimated dominance of up to 80% [45].

## Chapter 1

The necromass of enchytraeids and other soil animals is considered to be easily degradable [48], but excrement of soil fauna can actually be even more recalcitrant than ingested compounds, thereby fostering carbon sequestration. Despite these insights, the acknowledgement that excrement and necromass of soil organisms potentially contribute significantly to carbon sequestration remains largely unexplored. Therefore, we consider it to be a major knowledge gap regarding the functioning of heathland soil food webs (Table 1).

Despite extensive research illustrating the importance of soil fauna for processes such as SOM degradation, we currently still lack a fundamental mechanistic knowledge on their functional role for carbon cycling [74]. Although knowledge on food preferences of enchytraeid worms has been extensively synthesized [50], it still remains uncertain whether enchytraeids, and *C. sphagnetorum* in particular, *in situ* actively forage for fungal mycelium or bulk feed on SOM, as earthworms do in forests. Moreover, the extent to which they are able to assimilate various recalcitrant fungus-derived compounds (e.g. melanin) and carbon substrates and, hence, the differential contribution of various sources to their diet is unknown. Consequently, their functional role regarding carbon cycling within the heathland soil food web remains uncertain (Table 1).



**Figure 1:** Overview of carbon flow in the heathland soil food web whereby the considered uncertain links between groups are dashed. Circular illustrations (not to scale) from left to right: an ericoid mycorrhizal fungus (ERM) in symbiosis with its host plant, saprotrophic fungal (SF) mycelium and an enchytraeid worm. For the depiction of ERM and SF, figures were re-used with permission from Starrett *et al.* [79] and Crowther *et al.* [80] respectively.



## **Uncertainty regarding the effects of climate change on soil food webs**

Extreme climatic events such as prolonged drought and warming comprise one of the most important environmental change drivers affecting terrestrial ecosystems, especially in Western Europe [3]. As most soil organisms are sensitive to changes in soil water potential, soil moisture content is a key abiotic factor that determines their activity and community composition. Drought lowers heathland soil carbon influx in the short-term, as both root exudation [81] and litter production [82] are reduced. The long-term *in situ* experimental effects of drought on soil carbon stocks are highly variable in heathlands [52]. As such, the mechanisms governing context dependent responses to drought are very poorly understood, which adds to the uncertainties how strong extreme climatic events affect carbon sequestration.

Regarding impacts on soil organisms, drought induces osmotic stress which impedes reproduction and decreases activity and survival of enchytraeids [63]. Furthermore, drought might indirectly affect enchytraeids through altered availability of food resources [45]. However, reported global change manipulation effects on the diversity of heathland soil fauna are generally low [44]. Drought also affects soil microbial community structure by selecting for drought-tolerant species [83] and decreasing enzymatic activities involved in the decomposition process [84]. Further, while drought causes osmotic stress and lowers growth rates of fungi, they are generally more resistant to drought than bacteria because of their thick cell walls and more conservative growth strategies [83].

In general, differences in stress tolerance are relatively well studied within functional or taxonomic groups [85] but less so between functional groups and across trophic levels [86]. However, Franken and colleagues [86] for example found high interspecific variation in temperature tolerance among trophic levels in a soil arthropod community, which potentially causes trophic mismatches during extreme events. We expect a similar difference in sensitivity of functional

groups for drought and this adds to our limited understanding of the fundamental mechanistic link between stress-induced changes in food web composition, and net changes in soil carbon budgets. Therefore, we consider the stress sensitivities of soil organisms, especially fungi and enchytraeids that dominate the soil food web of heathlands, as an important knowledge gap (Table 1). For example, drought might indirectly foster sequestration of carbon by selecting stress-tolerant fungal species that are often highly melanized [77], since melanized fungal biomass decomposes slower than hyaline fungal biomass [23]. This critical dual role of melanin in both drought stress sensitivity and decomposability illustrates that functional traits of fungi driving susceptibility to environmental stressors are not necessarily independent from traits driving ecosystem processes.

**Table 1:** Heathland soil food web uncertainties

Uncertainty	Description	Explanation
Decomposer's input to SOM	The proportion of SOM that is derived from fungi and soil animals is unknown due to the uncertainty regarding the rate of recalcitrant carbon flow of dead fungi and animal faeces to the SOM pool	Fungi and soil animals exert both a degradation and a stabilization effect via conversion of assimilated solubles and polysaccharides into more recalcitrant polyphenolic compounds in their fungal tissues, which enter the SOM pool upon mortality or as excrements
Consumer's feeding behaviour	The ratio of fungi vs. organism-derived carbon substrates in the diet of enchytraeid worms is unknown	It remains uncertain whether enchytraeids in situ actively forage for fungal mycelium or bulk feed on SOM. Given the difference in the C/N ratio of fungi vs. SOM, this uncertainly largely contributes to the net effect of Enchytraeidae on carbon sequestration
Stress sensitivities of decomposers and consumers	The stress sensitivities of heathland soil fungi and enchytraeids to common abiotic stresses such as an increase in the frequency, duration and amplitude of heat waves and dry spells are unknown	Knowledge on stress sensitivities of different functional groups of soil organisms remains scarce, limiting our understanding of the fundamental mechanistic link between stress-induced changes in food web composition and net changes in soil carbon budgets

## **Modelling drought stress effects on carbon sequestration**

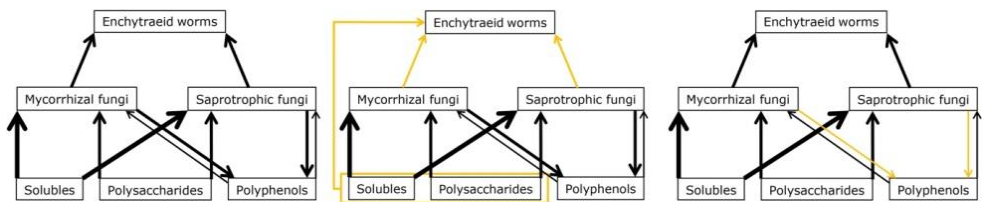
Given the multitude of uncertain factors identified above and summarized in Table 1, it is a challenge to quantify how these factors modify the impact of climate change on soil carbon sequestration. Disentangling these factors and quantifying their potential impact on carbon cycling is an important task because: (1) it enhances a mechanistic understanding of the role of food web ecology for carbon sequestration; and (2) it pinpoints those factors for which reducing uncertainty is most critical to enhance predictive capacity. Here, we implement drought stress effects in a well-known food web simulation model and inspect the implications of current uncertainties regarding soil food web structure and dynamics for predicting the effect of climate change on carbon sequestration in heathlands.

### **Parameters and simulations**

To assess how uncertainty in some of the assumptions behind soil food web models impact carbon cycling, we constructed a food web model based on the presence of dominant functional groups in heathlands. A Lotka-Volterra-based simulation model was structured and parameterized based on the model from Eklöf and Ebenman [87], but where necessary adapted to the heathland soil food web (Panel 2; SI Table 1 and Panel 1). The growth of basal functional groups is determined by their intrinsic growth rate, competition with other basal groups, and losses due to grazing. Consumers and predators grow when gains through grazing are larger than losses through mortality. The food web structure is encoded through a food-web matrix, listing who eats whom. The model uses plant litter as an input into three carbon pools and predicts community dynamics (i.e. the abundance of all groups through time).

To assess the impact of drought on soil carbon sequestration, no, low, medium and high drought stress were modelled using a 0%, 10-30%, 40-60%, and 70-90% reduction of fungal decomposition and soil fauna grazing rates. We simulated the effects of the four drought stress levels on soil carbon contents for

a total of nine scenarios (three x three), whereby each scenario represents a unique combination of uncertainties related to food web structure (three levels) and functional group stress sensitivity (three levels). Based on our review of the literature, we identified one 'default' food web structure and two variant structures that both illustrate a specific important uncertainty (Fig. 2). In the default food web structure, enchytraeids consume only fungi, and fungi have a large contribution to the polyphenolic carbon pool. In the second food web, enchytraeids bulk feed on SOM, but not on fungi, while fungi have a strong effect on SOM degradation. In the third structure, fungi contribute little to the polyphenolic carbon pool, and enchytraeids consume only fungi. Within each of these three food web structures, three different scenarios of stress sensitivities were simulated, giving a total of nine scenarios: i) fungi and enchytraeids were equally sensitive to drought stress; ii) fungi were more sensitive than enchytraeids (with no reduction of enchytraeid grazing rates); and iii) enchytraeids were more sensitive than fungi (with no reduction of fungi decomposition rates). For each scenario, 1000 simulations were run until equilibrium was reached.



**Figure 2:** Overview of the three different food web structures considered. First structure: enchytraeids are fungivorous, and fungal input to polyphenolic carbon pool is large. Second structure: enchytraeids are bulk SOM feeders, and fungal input to polyphenolic carbon pool is large. Third structure: enchytraeids are fungivorous and fungal input to polyphenolic carbon pool is low. Arrow thickness depicts the rate of carbon flow. Default structure in black, uncertainty related differences in yellow.

**Panel 2: Food web model characteristics**

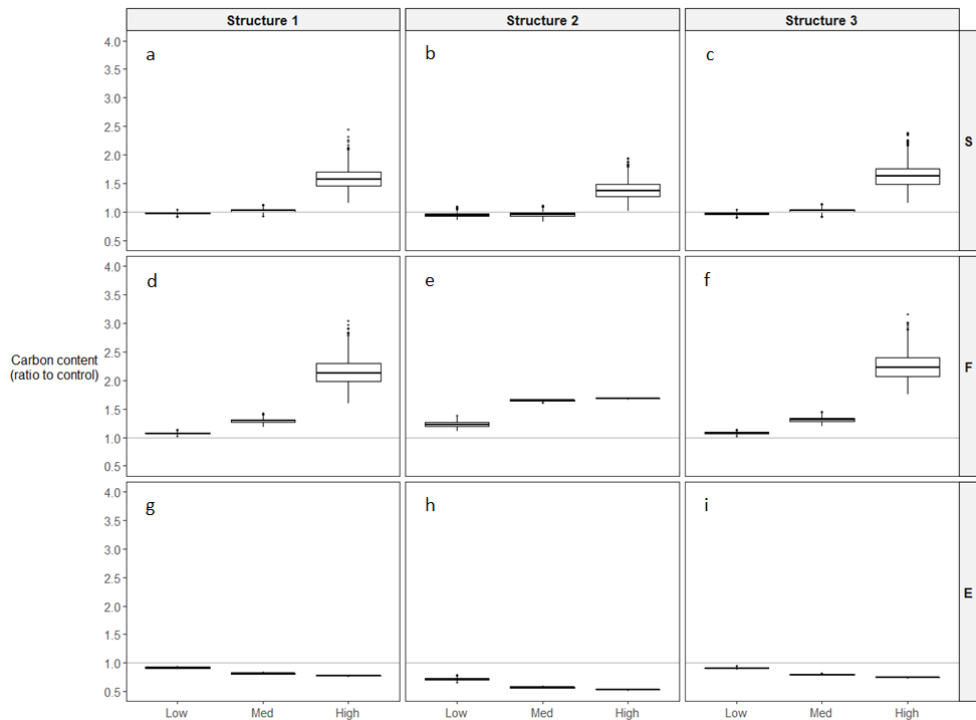
We described food web dynamics by a generalized Lotka-Volterra model as in Eklöf and Ebenman [87]. The change in population density through time ( $dx_i/dt$ ) of each functional group ( $i$ ) of the food web is described by its density ( $x_i$ ), multiplied by the sum of its intrinsic per capita growth rate ( $b_i$ ) and the interactions with all other components ( $S$ ) of the food web. These interactions are described as the per-capita effect ( $a_{ij}$ ) of the other functional group ( $j$ ) on the focal functional group ( $i$ ) multiplied by the density of the other functional group ( $x_j$ ). We have added a density-independent addition ( $A_i$ ) to the focal functional group to represent plant derived carbon input.

$$\frac{dx_i}{dt} = x_i \left( b_i + \sum_{j=1}^S \alpha_{ij} x_j \right) + A_i \quad \text{for } i = 1, \dots, S.$$

The interaction effect of functional group  $j$  on functional group  $i$  ( $a_{ij}$ ) is negative when  $j$  consumes  $i$  and positive when  $j$  is consumed by  $i$ . Multiple negative consumption effects of a consumer on different prey (or resources) are weighed based on the relative strength of the interactions with a total effect of -0.5. Opposite interaction strengths, the positive effects of prey on consumers ( $a_{ji}$ ), are derived from the  $a_{ij}$  interaction value by:  $a_{ji} = -e \cdot a_{ij}$ , with 'e' representing the assimilation efficiency with which prey biomass is converted into consumer biomass. Soil carbon contents are expressed as the sum of the three SOM components (solubles, polysaccharides and polyphenols).

## Results

Our results show that a difference in the sensitivity of fungi and enchytraeids to drought stress was more important than food web structure for predicting drought stress effects on carbon sequestration. Drought increased carbon sequestration when fungi were more sensitive than enchytraeids (F) or when both were equally sensitive (S) (Fig. 3 panels a-f). Drought stress decreased carbon sequestration when enchytraeids were more sensitive than fungi (E) (Fig. 3 panels g-i). Moreover, our food web simulations show that the feeding behavior of enchytraeids affected the extent of these sensitivity dependent stress-induced changes. When enchytraeids only fed on fungi and were less sensitive for drought than fungi (Fig. 3 panel d), their abundance reduced along with the stress-induced decrease in fungal abundance (as they had no alternative food source), resulting in the same outcome as for equal sensitivity (Fig. 3 panel a). However, if enchytraeids fed solely on SOM when being less sensitive for drought than fungi (Fig. 3 panel e), access to readily available carbon substrates allowed them to increase in abundance despite the decrease in fungal biomass. This resulted in a higher stress-induced carbon sequestration increase by enchytraeids compared to them being solely fungivorous (Fig. 3 panel d) or having the same drought sensitivity as fungi (Fig. 3 panel b). The rate of carbon flow from fungi to the polyphenolic carbon pool shows only a minor impact on stress-induced carbon sequestration changes (Fig. 3: panels a, d and g are very similar to panels c, f and i).



**Figure 3:** Model simulation results showing the effect of different degrees of drought stress (low, medium and high) on soil carbon contents, expressed as the ratio of the carbon content in the drought stress simulation over the carbon content in the control simulation (no reduction of grazing rates). Nine different cases (a-i) are shown in separate panels: structures 1, 2 and 3 with the three different scenarios of stress sensitivities (same sensitivity (S); fungi more sensitive (F) and enchytraeids more sensitive (E)).

### Food web complexity

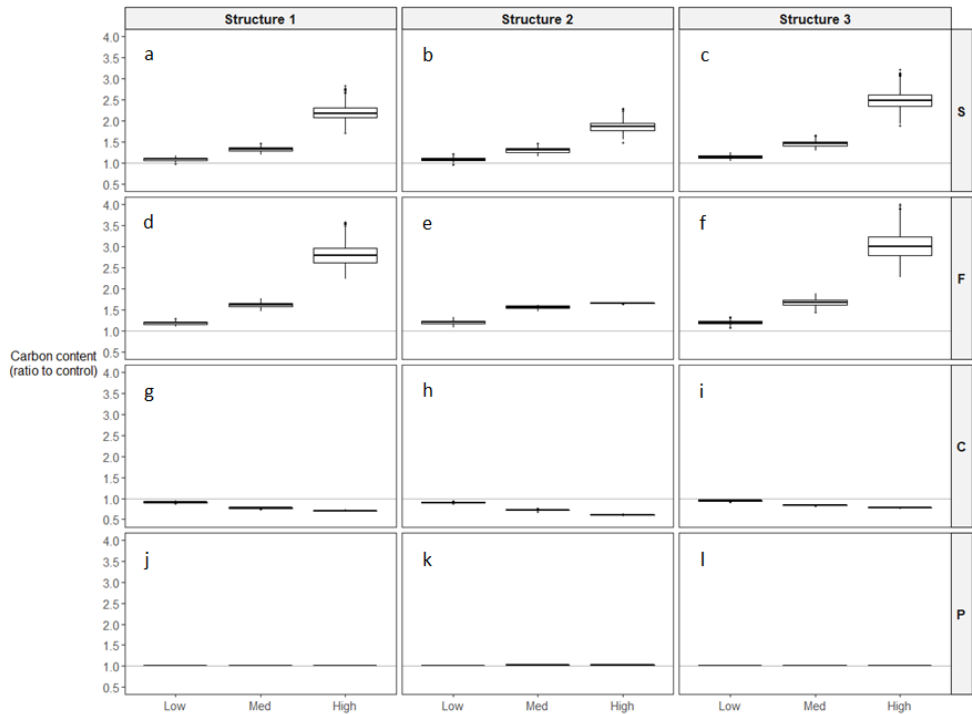
Our results illustrated that even a very simple food web already has so many uncertainties in some of its assumptions that, based on the currently available data, it is very difficult to make accurate predictions on the responses of soil carbon sequestration to future environmental changes. However, soil food webs can even be much more complex for other ecosystems than for heathland soils [88], [89], and there are parts of heathland soil food webs (e.g. predators) that we did not consider so far. For this reason, we repeated the simulations using a more comprehensive representation of heathland soil food web, by including less dominant functional groups of consumers (springtails and saprophagous mites)

and arthropod predators; such as predaceous mites, spiders and predatory beetles (SI Fig. 1).

This more complex food web was structured and parameterized in the same way as previously for the food web based on the dominant functional groups only (SI panel 2). Moreover, for optimal comparison, the same three variations of food web structures are considered, comprising the same two major uncertainties: i) degree of direct SOM consumption of consumers and ii) degree of feedback to the SOM pool of fungi and fauna. Within these three different structures, sensitivity uncertainty is again captured by modelling different sensitivity scenarios: i) all groups having the same drought sensitivity (S), ii) fungi (F), iii) all consumers (C) or iv) predators (P) are more drought sensitive than the other trophic levels, leading to a total of twelve different scenarios.

For this more complex food web (Fig. 4), drought-induced changes in soil carbon content showed a similar trend among all different scenarios but were even more variable than for the food web based on the dominant functional groups only (Fig. 3). For example, the high increase in carbon sequestration when fungi were more drought-sensitive and, together with the fauna, feed back to the SOM pool, was augmented from 240 to 300% (Fig. 3 panel f compared to Fig. 4 panel f). This quantitatively illustrates that the predictability of the effect of climate change on soil carbon sequestration decreases when food web complexity increases, as it implies making even more assumptions based on uncertain parameters. Thus, while even our simulations including additional consumers and predators are a simplistic representation of reality (as any model is by definition), this only strengthens our point that limited knowledge about soil food webs strongly limits our understanding of how soil carbon stocks will respond to climate change.





**Figure 4:** Model simulation results of the more complex food web. Twelve different cases (a-l) are shown in separate panels: structures 1, 2 and 3 with the four different scenarios of stress sensitivities (same sensitivity (S); fungi more sensitive (F); consumers (enchytraeids, springtails and saprophagous mites) more sensitive (C) and arthropod predators (P) more sensitive). Drought induced soil carbon content changes are similar but more variable than those for the standard food web complexity (Fig. 3).

### Model complexity

The role of food web structure within carbon cycling can be mathematically modeled using approaches of varying ecological scales and physiological mechanisms and consequently varying complexities. Hence, several potential model additions or different approaches should lead to a more comprehensive representation of (heathland) soil food web functioning. For example, consideration of the effects of drought stress on plant community composition and functioning increases robustness of the model by not only assessing the climate change induced changes in output of the soil system, but thus also considering changes in carbon input. Additionally, incorporating the effects of

drought on mortality rates in addition to decomposition and grazing rates increases representation of the model. The latter would likely make the effects of drought on soil carbon sequestration even more complex and variable. Other additions include: legacy effects of drought, nitrogen (N) mineralization, evolutionary adaptations and interactions within functional groups, as for example metabolically flexible generalist species can dominate during disturbances [90]. However, small-scale models capturing fundamental ecological mechanisms without excessive (mathematical) complexity are crucial before up-scaling towards global predictive models [65].

In line with an increased food web complexity, an increased model complexity entails more assumptions based on unknown parameters, thereby reducing tractability, robustness and potentially predictive capacity. For example, using a model with more parameters than ours, Berg *et al.* [88] found up to two-fold differences between measured and simulated carbon mineralization rates in a pine forest soil. Thus, for both food web complexity and model complexity, a balance needs to be found between tractability and realism, because even relatively simple models, such as in this study, require more understanding of soil food webs to accurately predict quantitative and even qualitative responses of soil carbon sequestration to increased droughts.

## **Conclusions**

The importance of soil food web structure and community diversity for ecosystem processes has been extensively illustrated by both theoretical and empirical studies [67], [68]. Therefore, adding of soil organisms in carbon simulation models may improve our assessment of the climate change mitigation potential of soils [69]–[74]. However, we demonstrate that stressed food webs of varying structural complexities can both increase and decrease soil carbon sequestration in heathlands, depending on differential stress sensitivities of and trophic links between consumers and decomposers. In addition, our results show that when food web structures differ among heathland sites, for example because of spatiotemporal variability [91], we can expect highly contrasting local or regional effects of climate change on carbon sequestration.

Thus, our results highlight that, even for a relatively uncomplicated system with a few dominant functional groups and a simulation model with a few but essential parameters, quantification of the relative stress-sensitivities of functional groups and how and to which extent these interact is needed in order to improve the forecast of carbon cycling models by adding the biotic drivers. As these uncertainties are potential important aspects among a variety of soils worldwide [92], we argue that more empirical research on these properties, in combination with simple mechanistic models such as ours, could potentially enhance understanding in other ecosystems as well.

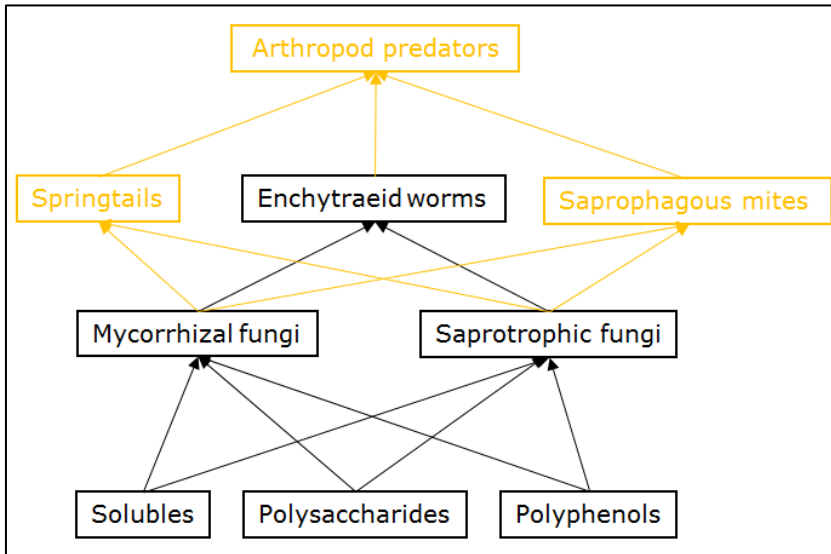
## Supplementary Information

**Table 1:** Model parameterization (based on references described in main text). Values attributed to different parameters for solubles (S), polysaccharides (Ps), polyphenols (Pp), ericoid mycorrhizal fungi (ERM), saprotrophic fungi (SF) and enchytraeids (Ench) are shown and explained. Values of the default  $\alpha$ -matrix are shown.

Parameter	Meaning	Values with explanation																																																	
$X_{init}$	Initial density	<table><tr><td>S</td><td>Ps</td><td>Pp</td><td>ERM</td><td>SF</td><td>Ench</td></tr><tr><td>0.75</td><td>0.75</td><td>1</td><td>0.5</td><td>0.5</td><td>0.3</td></tr></table> <ul style="list-style-type: none"><li>Carbon pools input ratio at 3:3:4.</li><li>Fungal guilds have similar abundance.</li><li>Enchytraeids are less abundant than fungi.</li><li>Equilibrium is however independent of initial density.</li></ul>	S	Ps	Pp	ERM	SF	Ench	0.75	0.75	1	0.5	0.5	0.3																																					
S	Ps	Pp	ERM	SF	Ench																																														
0.75	0.75	1	0.5	0.5	0.3																																														
$b_i$	Intrinsic per capita growth rate	<table><tr><td>S</td><td>Ps</td><td>Pp</td><td>ERM</td><td>SF</td><td>Ench</td></tr><tr><td>0</td><td>0</td><td>0</td><td>-0.5</td><td>-0.5</td><td>-0.5</td></tr></table> <ul style="list-style-type: none"><li>No growth rate of carbon pools due to <math>A_i</math>.</li><li>Negative growth of fungi and enchytraeids, representing natural mortality in the absence of resources and prey respectively.</li></ul>	S	Ps	Pp	ERM	SF	Ench	0	0	0	-0.5	-0.5	-0.5																																					
S	Ps	Pp	ERM	SF	Ench																																														
0	0	0	-0.5	-0.5	-0.5																																														
$A_i$	Density independent addition	<table><tr><td>S</td><td>Ps</td><td>Pp</td><td>ERM</td><td>SF</td><td>Ench</td></tr><tr><td>0.75</td><td>0.75</td><td>0.25</td><td>0</td><td>0</td><td>0</td></tr></table> <ul style="list-style-type: none"><li>3:3:1 <i>Calluna vulgaris</i> litter carbon composition ratio.</li></ul>	S	Ps	Pp	ERM	SF	Ench	0.75	0.75	0.25	0	0	0																																					
S	Ps	Pp	ERM	SF	Ench																																														
0.75	0.75	0.25	0	0	0																																														
$a_{ij}$	Interaction coefficients matrix	<table><tr><td></td><td>S</td><td>Ps</td><td>Pp</td><td>ERM</td><td>SF</td><td>Ench</td></tr><tr><td>S</td><td>0</td><td>0</td><td>0</td><td>-0.4</td><td>-0.4</td><td>0</td></tr><tr><td>Ps</td><td>0</td><td>0</td><td>0</td><td>-0.09</td><td>-0.09</td><td>0</td></tr><tr><td>Pp</td><td>0</td><td>0</td><td>0</td><td>-0.01</td><td>-0.01</td><td>0</td></tr><tr><td>ERM</td><td>0.2</td><td>0.027</td><td>0</td><td>-0.1</td><td>0</td><td>-0.25</td></tr><tr><td>SF</td><td>0.2</td><td>0.027</td><td>0</td><td>0</td><td>-0.1</td><td>-0.25</td></tr><tr><td>Ench</td><td>0</td><td>0</td><td>0</td><td>0.05</td><td>0.05</td><td>-0.1</td></tr></table> <ul style="list-style-type: none"><li>40:10:1 decomposition ratio of carbon pools, equally for both fungal guilds.</li><li>Enchytraeids have no fungal guild consumption preference.</li><li>The strength of intraspecific competition (<math>a_{ii}</math>) is equal for fungi and enchytraeids.</li><li>Assimilation efficiencies are 0.5, 0.3, 0, 0.2 and 0.2 respectively.</li></ul>		S	Ps	Pp	ERM	SF	Ench	S	0	0	0	-0.4	-0.4	0	Ps	0	0	0	-0.09	-0.09	0	Pp	0	0	0	-0.01	-0.01	0	ERM	0.2	0.027	0	-0.1	0	-0.25	SF	0.2	0.027	0	0	-0.1	-0.25	Ench	0	0	0	0.05	0.05	-0.1
	S	Ps	Pp	ERM	SF	Ench																																													
S	0	0	0	-0.4	-0.4	0																																													
Ps	0	0	0	-0.09	-0.09	0																																													
Pp	0	0	0	-0.01	-0.01	0																																													
ERM	0.2	0.027	0	-0.1	0	-0.25																																													
SF	0.2	0.027	0	0	-0.1	-0.25																																													
Ench	0	0	0	0.05	0.05	-0.1																																													

**Panel 1: Modelling food web uncertainties**

- We varied enchytraeid feeding behavior in structure 2 by adjusting  $\alpha_{\text{Ench-ERM\&SF}}$  and  $\alpha_{\text{Ench-S\&Ps}}$ , with 50% of total consumption on S and Ps in a 4 to 1 ratio (Pp are considered too recalcitrant to digest).
- We modeled fungal contribution to the polyphenolic carbon pool by making a second matrix ( $\beta$ ), adding  $X_{\text{ERM/SF}} * \beta_{\text{ERM/SF-PP}}$  to the equation describing Pp only, with  $\beta = +0.02$  for high (structures 1 and 2) and  $\beta = +0.005$  for low (structure 3) contribution respectively.



**Figure 1:** Overview of the considered more complex food web structure. Extensions towards the normal structure are in yellow. The default structure is shown (without direct SOM consumption of consumers (enchytraeids, springtails and mites) and without feedback to SOM). Arrow thicknesses are for clarity not varied to depict rate of carbon flow.

**Panel 2: Complex food web parameterization;** additions to standard food web

- In the default structure; Ench, St (springtails) and mites do not directly consume nor feed back to SOM.
- In structure 2, additionally to the direct SOM consumption of Ench, St consume 80% fungi and 20% SOM and mites 60% fungi and 40% SOM. In contrary to enchytraeids, they actively consume Ps over S (80-20% ratio).
- In structure 3; ERM, SF, Ench, St (springtails), mites and Pred (predators) feed back to the SOM pool with a 60-40% Ps-Pp ratio for fungi and 80-20% S-Ps for fauna, with a total feedback of 10% consumption (total of 0.05).
- Additionally, faunal consumers in all structures have more access to SF than ERM depicted with a 80-20% consumption ratio.
- Assimilation efficiencies are 0.2, 0.2, 0.1 for St, mites and Pred respectively.
- Basal parameters:

	St	Mites	Pred
$X_{init}$	0.2	0.2	0.1
$b_i$	-0.05	-0.05	-0.02
$A_i$	0	0	0

- Default  $\alpha$ -matrix:

	S	Ps	Pp	ERM	SF	Ench	St	Mites	Pred
S	0	0	0	-0.4	-0.4	0	0	0	0
Ps	0	0	0	-0.09	-0.09	0	0	0	0
Pp	0	0	0	-0.01	-0.01	0	0	0	0
ERM	0.2	0.027	0	-0.1	0	-0.1	-0.1	-0.1	0
SF	0.2	0.027	0	0	-0.1	-0.4	-0.4	-0.4	0
Ench	0	0	0	0.02	0.08	-0.1	0	0	0.025
St	0	0	0	0.02	0.08	-0.1	-0.1	0	0.425
Mites	0	0	0	0.02	0.08	-0.1	0	-0.1	0.05
Pred	0	0	0	0	0	0.0025	0.0425	0.005	-0.1



# Chapter 2

## **Isolation method complementarity increases cultivation coverage of heathland soil fungi**

Wouter Reyns, Erik Verbruggen, Frederik de Laender, Fons van der Plas, Richard D. Bardgett, Natalie Beenaerts, Francois Rineau

*Scientific Reports*; submitted October 2019

WR and FR designed the experiment in consultation with all other authors. WR and EV performed the experimental work and analysed the data. WR wrote the main manuscript text, which was reviewed by all other authors.



## **Abstract**

The majority of microbial diversity remains inaccessible because it cannot be cultivated. This microbial uncultivability is known as the 'great plate anomaly' (GPA) and implies that a very large fraction of possible microbiological knowledge remains unexplored. We addressed this issue by testing four different isolation methods and seven cultivation media for their specificity and complementarity towards heathland soil fungi, thereby maximizing the amount of cultivated taxa. We found that all isolation methods, but not cultivation media, show a high degree of specificity and complementarity towards the isolated taxa and their function, whereby each method led to the isolation of at least two taxa that were not isolated by any other method. Moreover, a new taxon was cultivated after four weeks of incubation, illustrating the usefulness of long incubation times. In conclusion, our results advocate the use of complementary isolation methods combined with long incubation times to accomplish high cultivation efficiency. In order to further minimize GPA by the use of complementary isolation methods, we encourage similar more elaborated studies in other ecosystem types, eventually allowing for a better understanding of soil fungal ecology.

## Introduction

Soils and the microorganisms they contain play a key role in a multitude of ecosystem processes and the societal services that ecosystems provide. However, a vast majority of soil microbial diversity remains inaccessible because many individual taxa cannot be cultivated in the laboratory. This remarkable inconsistency between taxa present in a given system and the small subset that can be cultivated is known as the 'great plate anomaly' (GPA) [93]. Despite recent progress in the use of culture-independent molecular techniques, there remains a predominant knowledge gap in soil microbiology due to unavailability of these "hidden" taxa in laboratory experiments. Therefore, there are large gaps in our understanding of the role of different microbial taxa in driving soil ecosystem processes. Minimizing GPA is therefore considered to be an important challenge in microbiological research.

To overcome GPA, microbiologists have tried to develop new laboratory cultivation methods that enhance isolation efficiency, for example by better mimicking *in situ* conditions. These methods have resulted in a notable increase in microbial recovery, but the reasons why so many microbes do not grow on artificial media remain largely unknown [94]. For soil micro-organisms, and fungi in particular, several classic isolation techniques have been described. The two most well-known and used are the dilution plate method and soil plate method [95]. Two less frequently used methods are the root maceration method and the immersion tube method [96], [97]. A frequent way to induce additional variation in these isolation techniques is to alter the nutrient composition, complexity and pH of the growth medium used for cultivation.

These different isolation techniques are known to be selective for different fungi. Dilution plating is considered to favor heavily sporulating fungi, but it simultaneously allows rare, slow-growing taxa to escape competition from fast-growing taxa (e.g. 'mold' saprotrophs) when very diluted [98]. Nevertheless, fast-growing taxa can also be captured from the less diluted samples. The soil plate method is methodologically very different from dilution plating, as soil structure is retained because it is not submerged in water. This method has

## Chapter 2

been shown to select for different taxa than dilution plating [95]. However, specificity towards certain traits/taxa is not very apparent. Maceration (where epidermal root cells are weakened by soaking of root tips in water) targets root-associated fungi, including mycorrhizal fungi. Immersion tubes select for actively growing fungi as these are filled with sterilized soil and contain small holes allowing actively growing fungi to colonize the soil tubes. These soil samples are then processed by soil or dilution plating (see materials and methods).

Here, our goal was to test four isolation methods and seven cultivation media for their specificity towards soil fungal taxa, in order to maximize the potential number of taxa and functional groups cultivated. Hence, we investigated which techniques and media are complementary or redundant to one another in terms of retrieved taxa and are therefore most efficient to use. Moreover, we tested whether long incubation times leads to cultivation of novel, not earlier isolated taxa.

## Materials and Methods

### Sampling

The study system is a dry heathland ecosystem located in the National Park, Hoge Kempen, Belgium. The park consists of a mosaic of pine stands, lakes and dry and wet heathlands. In November 2015, a total of eight soil cores (8cm in diameter, 20cm deep) were extracted every five meters along two transects (four cores per transect) from a dry heathland area in a plot located south of the park (50°59'02.1"N, 5°37'40.0"E). These transects were parallel to the longest side of the plot. The plot site was mainly composed by *Calluna vulgaris* (90%) and some grasses (mainly *Molinia caerulea*), mosses and bare soil. The heather in the plot was six to eight years old (in its 'building phase'), after being managed by burning. Soil samples were immediately brought to the laboratory, passed through a 2mm sieve, homogenized, and all eight pooled to form a single composite sample.

We tried to isolate as many taxa as possible from the heathland soil. For this purpose, we used four different isolation methods: the soil plate method [95], the dilution plate method, the root maceration method and Gochenaur's (1964) modification of the immersion tube method (based on [96], [97], whereby soil instead of agar medium is used). The cost in terms of time investment of these methods are all relatively small, but with falling cost from maceration and immersion tubes to dilution plating and finally soil plating.

For the soil plate method, 30mg of soil was added to an empty petri dish, and 20ml of agar medium was poured on top and gently swirled, in order to disperse soil particles both within and on the medium. The medium was poured just above stiffing temperature. For the dilution plate method, which favours heavily sporulating fungi, 1g of soil was diluted into 20ml of sterile distilled water. We prepared five dilution series ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ). A volume of 250 $\mu$ l of this suspension was spread with a sterile cotton swab on top of solid medium in a petri-dish.

## Chapter 2

The maceration method was used to isolate root-associated fungi (especially mycorrhizal fungi). For this purpose, we used two different approaches. First, based on [100]: three *Calluna vulgaris* plants were collected and twenty roots were cut into pieces of 2cm. These were first washed (to 'dissolve' epidermal cells) under running tap water for two hours and then further by twenty serial washes of five minutes in sterile water (to avoid contamination). We noticed however, that the epidermal cells of the roots did not go in suspension; hence we inoculated the roots themselves on the growth medium (one root per plate). Second, we performed an alternative approach based on Perotto *et al.* [101], [102] wherein three other plants were unrooted and 50 tiny lateral roots (<1mm diameter) were washed under running tap water for 24h. Afterwards, they were surface sterilized for one minute in 20% household bleach. Next, they were rinsed twice with sterile water. Using a potter/grinder, all root segments were together homogenized and partly macerated. A volume of 250µl of cell suspension was plated on each growth medium.

Finally, we used the immersion tube method as a way to isolate actively growing taxa (while the three other methods allow the growth of both actively growing and fungal spores). A volume of 1l of dry heathland top soil (top 20cm from the same plot) was autoclaved four times, and its sterility checked on growth medium. Then this soil was transferred to 15ml falcon tubes until they were half-filled, and four holes were made in different directions and different heights in the lower part of the tube (in sterile conditions). These so-called immersion tubes were brought to the field in a sterile packaging, and incubated in the soil of the sampling plot for twelve consecutive days (November 2015). Soil was taken from the tubes and processed through soil and dilution plate methods, as described above.

For all four methods, seven different types of agar based growth media (varying in richness, complexity and carbon source) were used: water, soil, Modified Melin-Norkrans (MMN), Czapek-Dox with 0.5% yeast extract and finally three media based on Ingestad solution [103] and a different carbon source: 0.4% cellulose, 0.4% pectin and 0.4% lignin. The pH was adjusted to five in all media using 10% HCl. In a full factorial design, this lead to a total of 35 different

combinations of methods (as immersion tubes were treated by soil and dilution plating). Each isolation method was replicated five times per growth medium (for the soil medium only three due to practical issues). The plates were then wrapped with parafilm, to avoid too much dehydration (especially for long periods of incubation), incubated in the dark at 23°C, and were kept for up to eight months to allow late and slow-growing taxa to germinate [98]. Once a strain started to grow, it was transferred to a new Czapek-Dox plate and stored at 5°C. Plates were checked and new growing isolates transferred (at least) every other day.

Isolates were visually sorted based on their morphology (growth form, growth rate, colour and production of exudates) into "morphotypes". Each of these morphotypes was identified by DNA isolation and amplification and if successful, sequenced by Macrogen. DNA was isolated using the Qiagen DNAeasy PowerSoil isolation kit. Subsequent PCR was performed using the Roche Applied Science 'FastStart High Fidelity PCR System'. We used primer set ITS1F-ITS4 amplifying ITS1 and ITS2 or, as it often lead to more efficient sequencing, the primer set ITS86F-ITS4 amplifying only ITS2 [104]. Annealing temperatures used for the two primers pairs were 55 and 57 °C, respectively.

### **Data analysis**

All isolates identified at species/genus level (taxa) were attributed to a taxonomic group (division: Ascomycota, Basidiomycota or Mucoromycota (former Zygomycota [105]) and to a functional group, which are formed based on available literature knowledge and visual inspection. A first level of differentiation is their type of association with another organism. We further distinguished free-living isolates based on their growth form, growth rate and color (a proxy for degree of melanisation [61]). We classified identified fungi as: root-associated (unknown/undefined type of relationship with plant root), mycorrhizal, phytopathogen, entomopathogen, lichen, mold saprotroph, soil saprotroph, wood saprotroph, black yeast and hyaline yeast (Table 1). Other parameters apart from function and taxonomy are isolation specifics such as method, growth medium type and duration (time between inoculation and picking).

## Chapter 2

**Table 1:** Overview of the approach to classify the soil fungal isolates into functional groups.

Group	Association	Growth form	Growth rate	Color
<b>Root-associated</b>	Plant (unknown type)			
<b>Mycorrhizal</b>	Plant symbiotic			
<b>Phytopathogen</b>	Plant pathogenic			
<b>Entomopathogen</b>	Insect pathogenic			
<b>Lichen</b>	Algae symbiotic			
<b>Mold saprotroph</b>	/	Hyphal	Fast	
<b>Soil saprotroph</b>	/	Hyphal	Slow in soil	
<b>Wood saprotroph</b>	/	Hyphal	Slow on wood	
<b>Black yeast</b>	/	Cellular		Dark
<b>Hyaline yeast</b>	/	Cellular		Pale

We tested for a relationship between the used isolation method and the i) identity (taxon), ii) functional group and iii) taxonomic group of the cultivated fungi, using chi-square tests. Also for the growth medium, we tested for a relationship with the i) identity, ii) functional group and iii) taxonomic group of the cultivated fungi. Additionally, we tested for a relationship between the duration of inoculation and isolate i) identity, ii) functional group, iii) taxonomic group, iv) isolation method and v) growth medium.

## Results

Using all isolation methods, 227 fungal colonies were isolated and categorized into 80 'morphotypes'. Of these 80 morphotypes, 52 isolates (65%) were successfully identified up to species/genus level, belonging to 17 different taxa (Table 2). These fungal taxa are dominated by Ascomycota, followed by Mucoromycota and Basidiomycota (SI Fig. 1). Regarding functional groups, the isolated taxa were dominated by mold saprotrophs and root-associated fungi, followed by mycorrhizal fungi, black yeasts and unknowns, and finally hyaline yeasts (SI Fig. 2). No wood saprotrophs, soil saprotrophs, phytopathogens, entomopathogens nor lichens were isolated.

**Table 2:** Isolated taxa and the taxonomic and functional group they are attributed to, grouped by the latter (number rank is arbitrary). Different species of the same genus are considered as one.

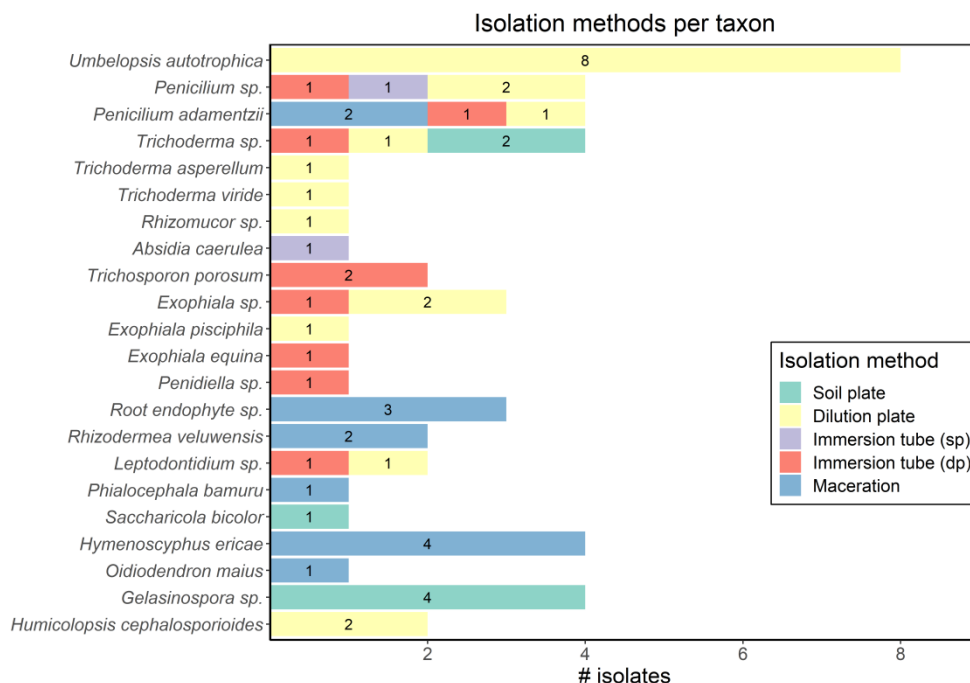
Number	Taxon	Functional group	Taxonomic group
1	<i>Gelasinospora sp.</i>	Unknown	Ascomycota
2	<i>Humicolopsis cephalosporioides</i>	Unknown	Ascomycota
3	<i>Oidiodendron maius</i>	Mycorrhizal	Ascomycota
4	<i>Hymenoscyphus ericae</i>	Mycorrhizal	Ascomycota
5	<i>Leptodontidium sp.</i>	Root-associated	Ascomycota
6	<i>Saccharicola bicolor</i>	Root-associated	Ascomycota
7	<i>Root endophyte sp.</i>	Root-associated	Ascomycota
8	<i>Rhizodermea veluwensis</i>	Root-associated	Ascomycota
9	<i>Phialocephala bamuru</i>	Root-associated	Ascomycota
10	<i>Exophiala sp.</i>	Black yeast	Ascomycota
11	<i>Penidiella sp.</i>	Black yeast	Ascomycota
12	<i>Trichosporon porosum</i>	Hyaline yeast	Basidiomycota
13	<i>Absidia caerulea</i>	Mold saprotroph	Mucoromycota
14	<i>Trichoderma sp.</i>	Mold saprotroph	Ascomycota
15	<i>Umbelopsis autotrophica</i>	Mold saprotroph	Mucoromycota
16	<i>Penicilium sp.</i>	Mold saprotroph	Ascomycota
17	<i>Rhizomucor sp.</i>	Mold saprotroph	Mucoromycota



## Isolation methods

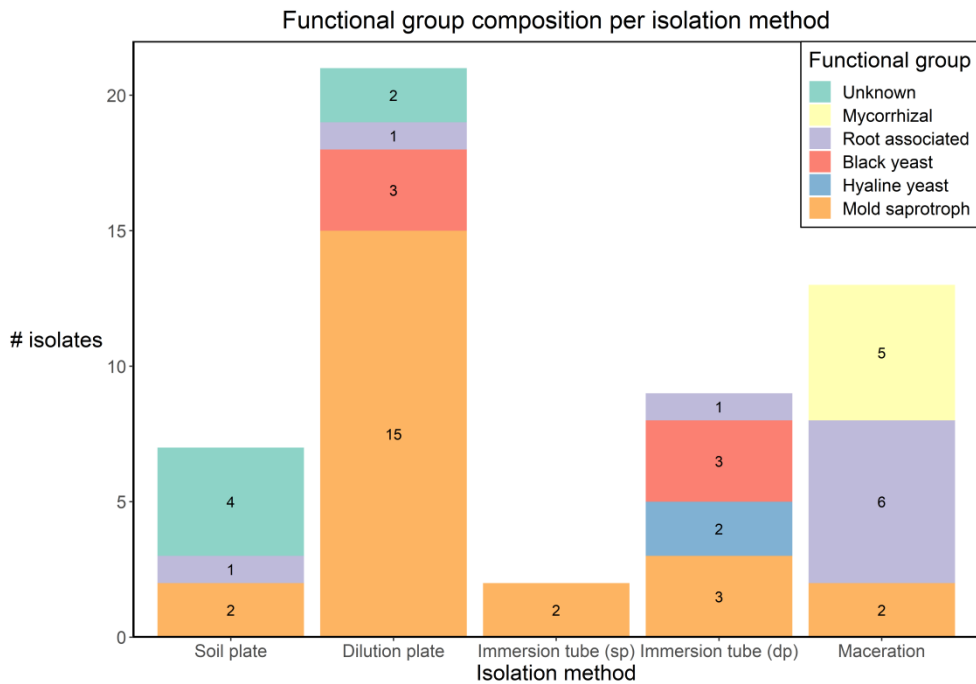
Most isolates were cultivated through dilution plating (21), followed by maceration (thirteen), immersion tubes with dilution plating (nine), soil plating (seven) and finally immersion tubes with soil plating (two). There was a significant relationship between isolation method and the identity of the cultivated isolates ( $P < 0.001$ ) (Fig. 1), its functional group ( $P < 0.001$ ) (Fig. 2) and its taxonomic group ( $P = 0.002$ ) (SI Fig. 3).

Regarding composition of taxa, there were several interesting observations (Fig. 1): *Umbelopsis autotrophica* (a mold saprotroph) was isolated only through dilution plating (eight); *Hymenoscyphus ericae* (four) (a mycorrhizal fungus), *root endophyte sp.* (three) (a root-associated fungus) and *Rhizodermea veluwensis* (two) (a root-associated fungus) were isolated only by maceration; and *Gelasinospora sp.* (unknown) was isolated only using the soil plate method.



**Figure 1:** The different methods of isolation by which all isolates of different taxa were cultivated (ordered per functional group in a reversed order compared to Table 2). Sp and dp refer to soil plate and dilution plate respectively. The methods show high taxon specificity, as the isolation of some taxa is completely method dependent.

Regarding functional group composition, all mycorrhiza (five) and six out of nine root-associated fungi were isolated by the maceration method (Fig. 2). A high fraction (fifteen out of 21) of mold saprotrophs was isolated using dilution plating. Black yeasts were isolated only through dilution plating (three directly, three through immersion tubes), whereas hyaline yeasts (*Trichosporon porosum*) were isolated only from immersion tubes with dilution plating (but only two cases). Hence, five out of eleven strains isolated by immersion tubes are yeasts (black or hyaline).



**Figure 2:** The proportion of different functional groups retrieved using different isolation methods. The isolation methods show high functional group specificity.

For all isolation methods, Ascomycetes were the dominant taxonomic group (SI Fig. 3). For the dilution plate method however, Mucoromycota are almost equally abundant than Ascomycetes. The only two Basidiomycetes (*Trichosporon porosum*) were isolated via dilution plating after immersion tube inoculation.



The duration of isolation after inoculation on the cultivation plates varied from two up to 44 days for the identified isolates, with a general trend of early isolation (with a peak at day four) (Fig. 3). However, there was much variation among taxa, functional groups and isolation methods. Several taxa were isolated early (less than ten days) only (e.g. both *Penicilium* taxa), late (more than ten days) only (e.g. all three *Exophiala* taxa) or both early as well as late (e.g. *Umbelopsis autotrophica* and *Humicolopsis cephalasporioides*). Black yeasts and mycorrhiza are in general isolated later (SI Fig. 5) than the average four days. Also the maceration method shows a later than average peak of isolation duration (SI Fig. 6).

Two unidentified (and hence not considered) isolates were isolated after exactly 200 days. Both colonies appeared on a Czapek-dox medium plate inoculated with a  $10^{-4}$  diluted soil solution retrieved from immersion tubes. Both isolates are very dark pigmented and resemble very closely the growth form of the identified *Exophiala* isolates, a black yeast. However, as we were not able to identify them, their identities remain unknown.

## Discussion

Cultured soil fungi were dominated by mold saprotrophs such as *Penicilium*, *Trichoderma*, *Mortierella* (*Umbelopsis*), *Mucor* and *Absidia* and mycorrhiza's such as *Hymenoscyphus ericae* and *Oidiodendron*. This is in line with two other fungal isolation studies in dry heathlands [30], [106], which respectively used only a soil washing technique and both soil plating and maceration. We also isolated rarer and less frequently cultivated taxa known to inhabit these types of soils [107], [108], such as *Exophiala* and *Rhizodermea veluwensis* (a black yeast and root-associated fungus respectively).

### Isolation methods

The isolation methods showed a high specificity towards the cultivated species/genus. This significant method-taxon relationship can be attributed to the functional group the taxon belongs to, as different isolation methods select for different functional traits the taxon possess. Moreover, the isolation methods showed a high degree of complementarity, whereby each method has led to the isolation of multiple taxa that were not isolated by any other method and hence would otherwise not have been cultivated.

Maceration almost exclusively isolated mycorrhizal and other root-associated fungi. Hence this very high specificity illustrates the effectiveness of our maceration protocol, which is in line with expectations as this method targets fungi present in and around roots. Although, it has been shown that there can still be a mismatch in composition between culturing and direct DNA extraction from the same ericoid mycorrhizal roots [109].

In this experiment, dilution plating selected for mold saprotrophs and yeasts. This is consistent with literature, as serial dilution plating is considered to be a way to allow rare or slow-growing taxa to escape competition from fast-growing taxa (e.g. mold saprotrophs) when very diluted [98]. Hence, we expected the heavily sporulating molds to be isolated from the low dilutions whereas the more slow-growing (black) yeasts are likely isolated from more diluted plates.

However, we were unfortunately not able to make this distinction as we did not take into account the dilution factor upon isolation.

Although soil plating can to a certain extent select for different taxa than dilution plating [95], specificity towards certain traits and hence functional groups is considered to be less pronounced for this method. Methodologically it differs from the other methods in the retaining of the soil structure (not submerged in water), which likely is essential for particular taxa to be able to grow, as fungi interact with soil particles in a variety of ways [110]. On the other hand, soil aggregates have to be broken open to avoid (physical) entrapment of microorganisms [111]. In our isolation analysis, the link between soil plating and *Gelasinospora* is apparent. Hence this appears to be an isolate that needs the soil structure to be cultivated, but due to the lack of knowledge on the ecology of this genus, we cannot explicitly explain its causes.

Yeasts compose a disproportionally high proportion of taxa isolated through immersion tubes. They can therefore be considered most active, as this method is designed for the isolation of actively growing fungi. This clearly illustrates that this method not necessarily selects for pioneer taxa such as mold saprotrophs (which could be expected as fast-growing molds could quickly and easily penetrate through the holes with their hyphae), but rather for the cellular growth type of yeasts, including slow-growing taxa such as *Exophiala*.

### **Isolation media**

In contrast to the isolation methods, there was a lack of specificity of all seven used growth media towards cultivated fungal taxa and the functional and taxonomic groups they were attributed to. Hence in contrast to expectations, variation in the overall nutrient richness and carbon source present does not affect the isolation of specific fungal taxa. As nitrogen likely is the nutrient that often limits growth of soil fungi in heathlands, varying nitrogen sources and contents could be a possible way to further increase diversity in growth media which would therefore more likely translate into diversity in isolated taxa. However, our water and soil growth media were free of (easy) nitrogen sources, but thus without affecting the identity of the isolated fungal taxa.

### Duration

The results of duration of isolation are in line of expectations considering the relationship between growth rate and function. In general, fast-growing heavily sporulating mold saprotrophs such as *Penicillium sp.*, which are mainly isolated by dilution plating, are isolated early. Also the isolates cultivated using soil plating, mainly *Gelasinospora*, were mostly isolated early. Slow-growing black yeasts, root-associated fungi and especially mycorrhiza such as *Hymenoscyphus ericae*, which are mainly isolated by respectively dilution plating (with and without immersion tubes), maceration and again maceration, are isolated later.

Moreover, the two strains isolated after 200 days confirms that serial dilution plating is a way to allow rare or slow-growing taxa to escape competition from fast-growing taxa (e.g. mold saprotrophs). However, for this extremely late case, we unfortunately cannot judge whether it is a novel taxon that has not been cultivated earlier, which has been questioned in another study [98]. Anyway, a less extreme case, the *Rhizomucor sp.* isolate which has been isolated after 28 days, shows that incubating plates for longer times than conventionally can lead to the cultivation of novel fungal taxa. As the generation time of fungi in natural soils is approximately 10 times larger than those of bacteria [112], long incubation times likely are even more important for soil fungi than for soil bacteria to isolate as many taxa as possible.

### Representation

We attributed isolated into different groups (morphotypes) based on several morphological characteristics (see Materials and Methods) before ITS sequencing one isolate of each morphotype. By doing so, we potentially did not identify some unique taxa, in the case that they were attributed to the wrong group (and not covered by another group). However, the fact that the 52 morphotypes identified based on ITS sequencing, represent 'only' 17 different taxa, indicates that the morphological parameters we used to divide isolates into morphotypes were very strict when grouping and chances are thus low that we missed a 'new' taxon.

Additionally, to put our isolation effort into perspective, we compared the composition of our isolated taxa with a small-scale culture-independent NGS (next generation sequencing) analysis. This analysis was however performed on soil samples of a plot adjacent to, and one year after, the sampling for isolation. Therefore, due to the large spatiotemporal variability in fungal community composition, this comparison has to be considered very carefully. Nevertheless, it does give some indication of the representability of our fungal culture. In this NGS analysis, 94 different fungal OTU's were distinguished out of approximately 9000 reads. Of these 94 OTU's, approximately 9% had been isolated. The GPA is estimated to be as high that only approximately 1% of present micro-organisms currently has been lab cultivated [94], [113]. In that regard, isolating 9% of the present soil fungi is relatively large. However, this is a habitat unspecific and bacteria-oriented estimation. But, due to the lack of studies that explicitly characterize soil fungal communities by isolation combined with a cultivation independent approach, it remains difficult to evaluate the isolation effort.

Anyways, there still is room for improvement to close the gap between *in situ* conditions and artificial laboratory conditions. Micro-organisms often require specific unknown exudates of other organisms to be able to grow [114]. In this regard, several new methods have been developed. For example, the identification and use of siderophores has been shown to increase bacterial cultivation efficiencies [115]. Additionally, the previously mentioned temporal variation in fungal community composition also means that isolating multiple times throughout the year would also have increased the output/diversity of isolated fungi. Combined with a NGS, this also already informs about the abiotic stress sensitivity of the taxa by linking to the environmental conditions/disturbances present during sampling.

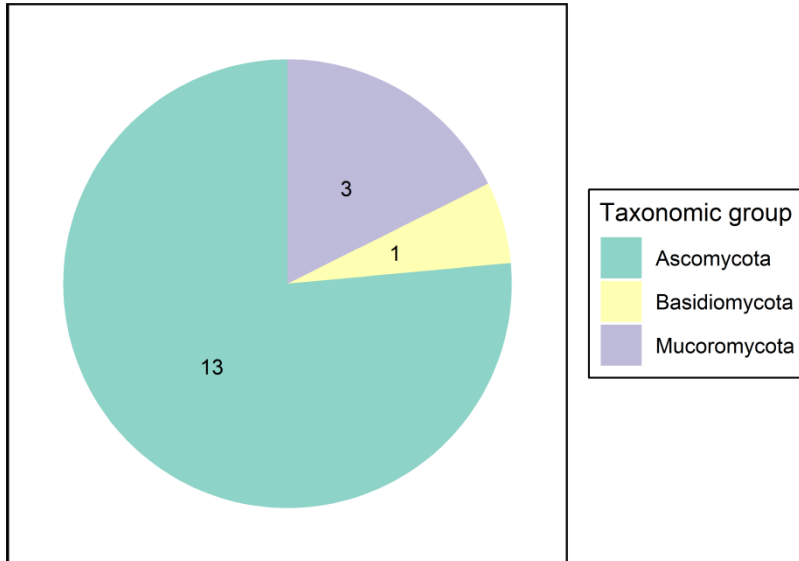


## Conclusions

Soil fungal isolation methods show high taxon specificity in line with their functional traits. Additionally, the four isolation methods tested showed a high degree of complementarity, whereby each method lead to the isolation of at least two taxa that were not isolated by any other method (including rarer and less frequently cultivated taxa). Also considering the time-cost of the different methods, the benefit in terms of extra taxa isolated is not outweighed by the extra time investment to perform them. Only when interested in a particular functional group, one can select methods that isolate the most taxa of interest for the specific goals of a certain study. In contrast to the isolation methods, varying the nutrient composition of the cultivation medium, in order to better mimic different *in situ* abiotic conditions, did not show taxon specificity and hence did not improve cultivation. Finally, our isolation experiment advocates the use of long incubation times, as new taxa can be cultivated after up to 28 (and potentially even 200) days of incubation. Based on this investigation, it remains speculative to state to what extent the use of different isolation methods explicitly overcome GPA. Nevertheless, we clearly illustrated high method specificity and, most interestingly, complementarity towards cultivated and *in situ* present fungal taxa. In order to minimize GPA, we advocate for more elaborated isolation studies that include a culture independent approach, which is crucial for a better understanding of soil ecosystem processes.

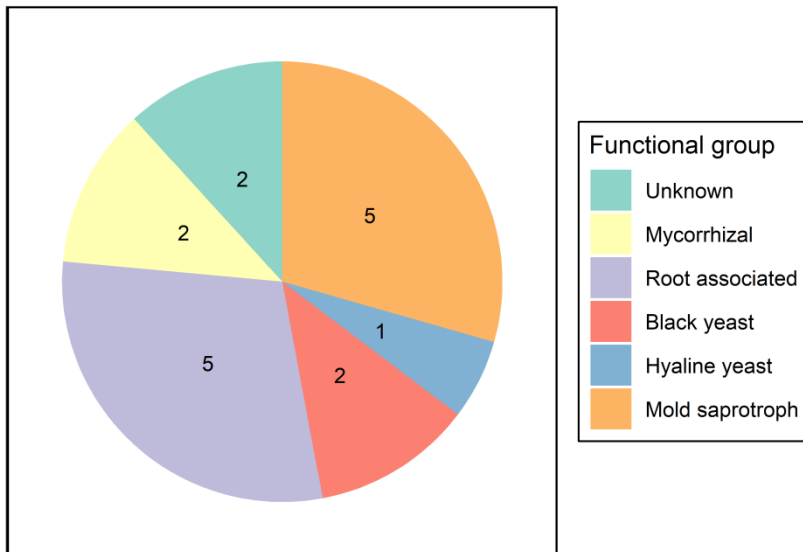
## Supplementary Information

Cultivated taxa taxonomic group composition

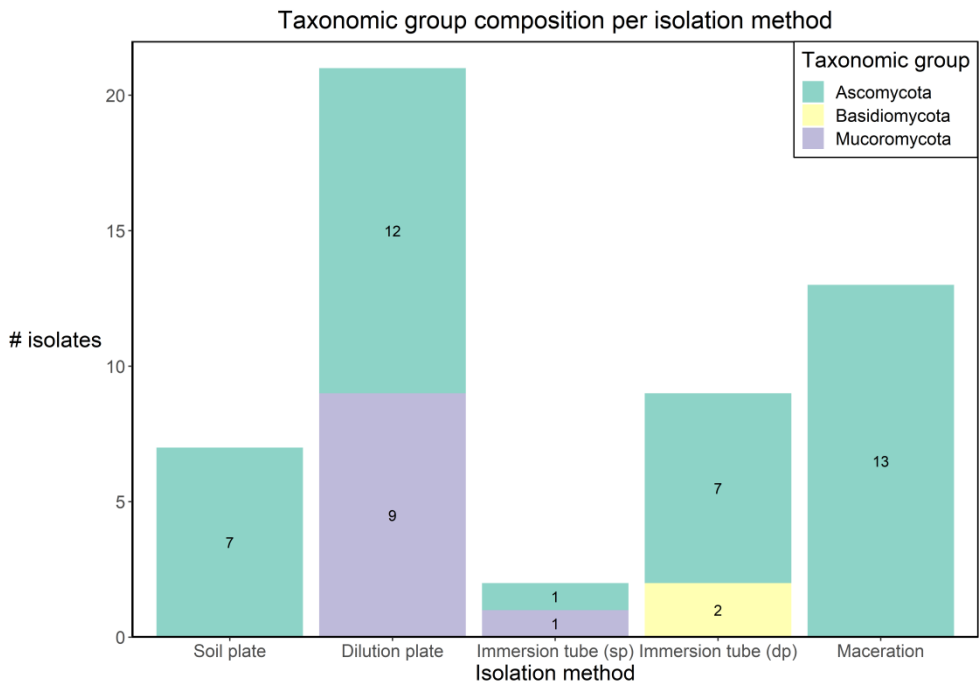


**Figure 1:** Taxonomic group composition of cultivated taxa (n=17).

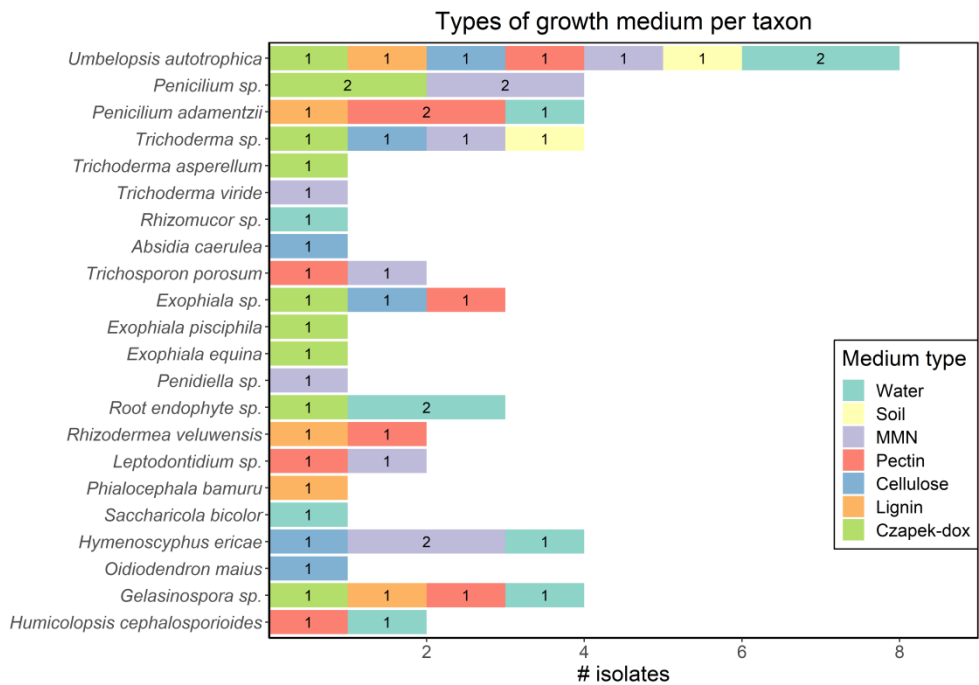
Cultivated taxa functional group composition



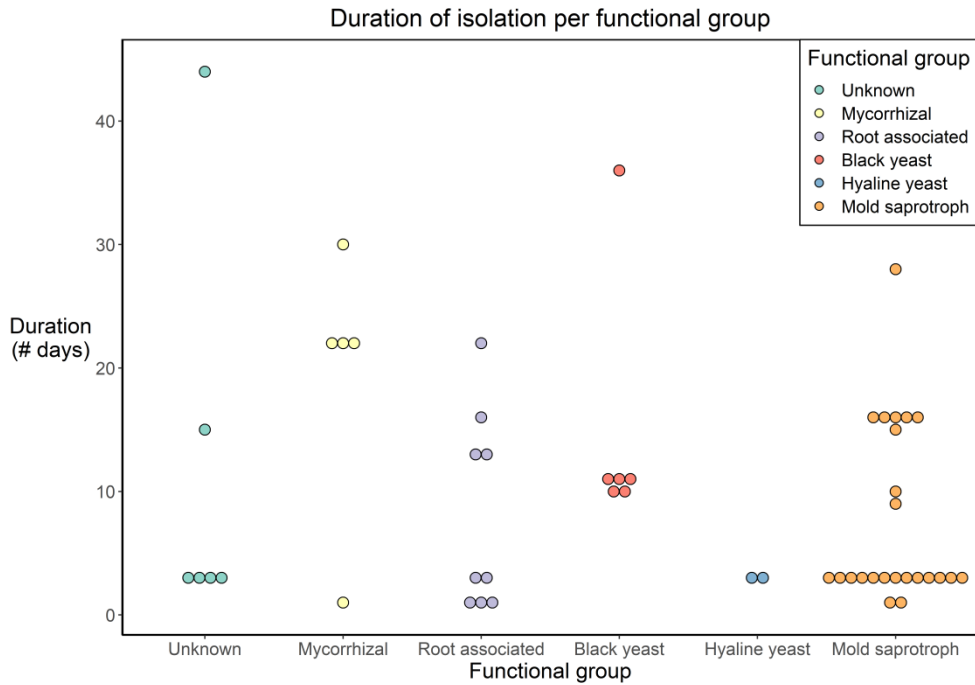
**Figure 2:** Functional group composition of cultivated taxa (n=17).



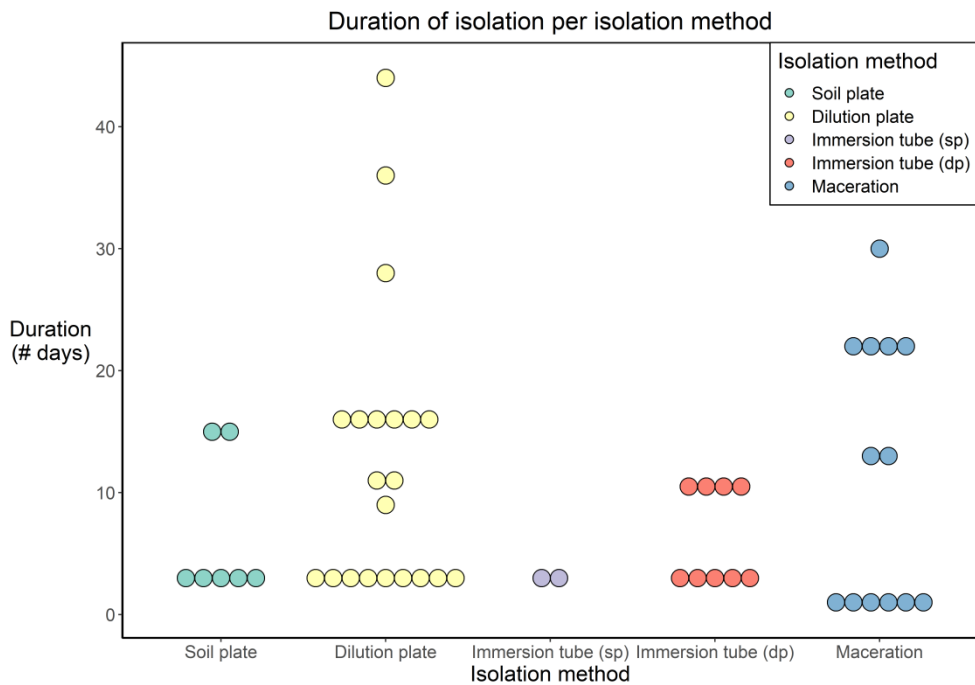
**Figure 3:** The proportion of taxonomic groups retrieved using different isolation methods.



**Figure 4:** The growth media by which all isolates of different taxa were cultivated.



**Figure 5:** The duration (in number of days) of isolation per functional group.



**Figure 6:** The duration (in number of days) of isolation per isolation method.



# Chapter 3

## **Fungal functional traits do not explain sensitivity to temperature and water stress**

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WR, FDL and FR designed the experiment in consultation with all other authors. The experiment was performed by WR and analyzed by WR, FDL, CC and FR. WR wrote the main manuscript text, which was reviewed by all other authors.

## **Abstract**

Trait-based approaches that functionally characterize individual species have been widely used in ecological research to advance understanding of how community shifts under environmental change affect ecosystem functioning. In the last decade, there has been an increased recognition of the potential of such approaches to inform soil fungal ecology. However, such frameworks for fungal ecology currently are mostly conceptual, and lack empirical validation. Here, we tested whether the sensitivity to temperature and water stress of a range of heathland soil fungal taxa could be predicted on the basis of a range of morphological and physiological functional traits, including intrinsic per capita growth rate, enzymatic capability, melanin content, hydrophobicity and mycelial density. We found that the abiotic stress sensitivity of soil fungal taxa was unrelated to any of the measured functional traits. This lack of relation between the stress sensitivity of fungi to temperature and water availability with functional traits questions the applicability and relevance of the considered traits for such frameworks. Hence, for the desired shift to trait-based approaches in soil fungal ecology to be effective, we advocate future studies to explore a wider range of traits associated with abiotic stress sensitivity in order to ultimately improve our predictions of how climate change will alter soil carbon sequestration through changes in soil fungal communities.

## Introduction

Terrestrial ecosystems and the various functions and services that they provide are under threat from human-induced climate change. Given this, there is currently much discussion regarding the potential to mitigate rising atmospheric CO<sub>2</sub> concentrations by the natural process of carbon sequestration into soil organic matter [6]. As such, a crucial question is whether climate change increases or decreases the capacity of soils to sequester carbon, and hence whether ecosystems will buffer or further accelerate climate change. Despite considerable research on this topic, no consensus has been reached on the fate of global soil carbon stocks under future climate change.

The importance of the soil biological community in regulating the effects of climate change on soil processes related to carbon sequestration has been extensively illustrated, whereby numerous studies show that shifts in soil community composition due to climate change can have significant consequences for soil carbon cycling [67], [68], [83], [116]. However, such *in situ* studies typically measure community shifts at a too high level of organization (for example broad functional or taxonomic group) to understand how it causes the observed changes in soil carbon contents. In the last decade, there has been an increased use of trait-based approaches to describe the assembly and functioning of soil fungal communities [77], [117]–[120], which would consequently lead to a better understanding and predictability of the role soils could play in climate change mitigation.

Fungal trait-based frameworks that can be used to link shifts in the soil fungal community in response to environmental changes, including drought and warming, with shifts in ecosystem functions, such as soil carbon storage [77], are often based on Grime's classic CSR framework [121], which has been widely used in plant ecology [122]. The CSR framework classifies species in a continuous trait space as competitors (C), stress tolerators (S), or ruderals/colonizers (R) based on their life history traits. A variety of morphological and physiological traits, including growth rate, growth form and the production of melanin, osmolytes, antibiotics, toxic secondary metabolites



### Chapter 3

and enzymes for degradation of various organic matter compounds such as cellulose and lignin, have been used to classify fungal taxa using the CSR framework [77], [117]–[120]. For instance, fungi are considered competitive if they show a high degree of antibiotic, toxic secondary metabolite and lignolytic enzyme production by capitalizing on resources in a productive environment. Fungal taxa are considered stress tolerators if they grow slowly and withstand unfavorable abiotic conditions by producing protective compounds such as melanin and osmolytes. Finally, species are considered as ruderal if they show high growth and dispersal rates by producing relatively high rates of hydrolytic enzymes, which should allow them to re-colonize and establish in environments facing frequent perturbations.

These fungal trait-based frameworks are currently mostly conceptual and based on literature, describing how various traits are assumed to vary with predefined life history strategies such as CSR. However, these descriptive frameworks have been rarely empirically validated. Therefore, we investigated whether several key soil fungal traits relate to their sensitivity to increased temperature and drought (water stress). We considered traits that have been put forward in the literature as important functional traits, several of which are expected to directly relate to abiotic stress sensitivity. Specifically, we tested whether growth rate, mycelial density, hydrophobicity, melanin content, color ('darkness' as a proxy for melanin content) and production of three hydrolytic enzymes and three (per)oxidative enzymes predict the growth response of soil fungal taxa to temperature and water stress.

The optimal temperature for growth of fungi in temperate soils generally is around 25–30°C, with the maximum temperature for possible growth usually being around 10°C higher [112]. These optimal growth rates are off course much higher than those generally realized under different environmental conditions *in situ*. Fungi were grown at 23°C, a standard temperature for cultivating heathland soil fungi [106], and exposed to mild (28°C) and high (35°C) increases in temperature, which can thus be considered as mild and high temperature stress respectively. Water stress was simulated in an acute way

prior to the experimental period in which temperature stress was simulated (see further).

We expected fungal growth rates to be generally negatively affected by temperature and water stress [57]. Additionally, we expected the considered traits to partly explain variations in stress responses among taxa. Melanin is a pigment with a well-known important protective role in stress tolerance [61]. Additionally, hydrophobicity and density, two morphological mycelial traits that are hypothesized to be informative for fungal ecology [117], were also expected to be important for the tolerance of fungi to abiotic stress [123]. Production of (per)oxidative enzymes is linked with competitive ability and we therefore expected that fungi that produce these enzymes are generally also relatively stress tolerant. In contrast, fungi that show high capacities of hydrolytic enzyme production are considered fast growing ruderal species that generally are considered more sensitive to stress [77]. In conclusion, we expected melanin, color darkness (proxy for melanin), hydrophobicity, density and (per)oxidative enzymes production to be positively related with stress tolerance and we expect growth rate and hydrolytic enzymes production to be negatively related with stress tolerance.

These hypotheses were tested using seventeen fungi cultivated from soils of semi-natural heathland, which have fungi-dominated soil systems of high soil carbon content, thereby representing potential locations for soil carbon sequestration. The effect of climate change on heathland carbon stocks, however, is uncertain: studies report highly variable effects of drought on carbon sequestration across sites, with a tendency for increased sequestration (carbon sink) at drier sites and decreased sequestration (carbon source) at wetter sites [52], [53], whereby the underlying mechanisms of these changes are poorly understood. Therefore, applying trait-based approaches could be an effective way to link shifts in the soil fungal community in response to drought and warming, with changes in heathland soil carbon storage.

## Materials and Methods

### Set-up

Seventeen heathland soil fungal isolates, that were cultivated from a dry heathland soil by Reyns *et al.*, *submitted* (chapter 2), were exposed to temperature and water stress in petri-dishes. The isolates belong to fourteen different taxa (at the species/genus level) (table 1), whereby different isolates of the taxa *Trichoderma*, *Umbelopsis autotrophica* and *Leptodontidium* showed different intraspecific growth responses in a priori trials and where therefore exposed in duplicate. The experimental petri-dish growth medium consists of basic Ingestad nutrient solution [103] with glucose (0.4%) as a carbon source and agar (1%) as a stiffening agent. The pH of the medium was lowered to five using 10% HCl. 20 ml of growth medium was poured in a 10 cm diameter petri-dish and topped with a cellophane sheet to avoid penetration of the medium by the fungal mycelium. Isolates were grown at control conditions (see further under 'Design') on the experimental medium for two successive generations prior to the start of the experiment.

**Table 1:** Investigated fungal taxa and the order and division (taxonomic group) they belong to.

<b>Taxon</b>	<b>Order</b>	<b>Division</b>
<i>Penidiella sp.</i>	Capnodiales	Ascomycota
<i>Exophiala sp.</i>	Chaetothyriales	Ascomycota
<i>Hymenoscyphus ericae</i>	Helotiales	Ascomycota
<i>Leptodontidium sp.</i> (2)	Helotiales	Ascomycota
<i>Phialocephala bamuru</i>	Helotiales	Ascomycota
<i>Trichoderma sp.</i> (2)	Hypocreales	Ascomycota
<i>Saccharicola bicolor</i>	Pleosporales	Ascomycota
<i>Gelasinospora sp.</i>	Sordariales	Ascomycota
<i>Humicolopsis cephalosporioides</i>	<i>incertae sedis</i>	Ascomycota
<i>Root endophyte sp.</i>	/	Ascomycota
<i>Trichosporon porosum</i>	Tremellales	Basidiomycota
<i>Absidia caerulea</i>	Mucorales	Mucoromycota
<i>Rhizomucor sp.</i>	Mucorales	Mucoromycota
<i>Umbelopsis autotrophica</i> (2)	Mucorales	Mucoromycota

## Design

Water availability was set at two levels: absence (control) and presence of water stress, which was simulated by transferring a plug of mycelium to an empty petri-dish 72h prior to the start of the experiment. Normal and dried mycelial plugs of three by three mm were then transferred to the middle of a new experimental petri-dish and exposed for 30 days to either of three different temperature levels: 23°C (control), 28°C (mild temperature stress) and 35°C (high temperature stress). Hence, in a full-factorial design, six treatments were considered. Water stress was thus simulated in an acute way (72h) prior to the 30 day experimental period. During this experimental period, moisture recovery took place, whereby resilience rather than resistance to water stress was quantified. For each treatment of each of seventeen fungal isolates, three replicates were performed, whereby one was further analyzed because of high consistency among replicates.

## Trait quantification

Fungal growth was quantified for 30 days by measuring colony surface area from images of the petri-dish surface. Petri-dishes were scanned daily in the first week, every second day in the second week and twice per week for the remaining experimental period, leading to a total of 14 timepoints. From the scans, we quantified fungal growth rates under all six treatments and calculated their growth stress response (see further under 'Quantification of sensitivity'). The scans were also used to quantify the color of the fungal mycelium (average over time over all treatments). Additionally, dry biomass was measured after the 30 day exposure period, to quantify the density of the mycelium, which was also considered as a possible trait relating with stress sensitivity.

In a separate experiment, we measured enzymatic capabilities (under control conditions) of the same seventeen fungal isolates. Fungi were cultured in liquid medium containing Ingestad nutrient solution, glucose and sterilized soil (five biological replicates per isolate), the latter to trigger a broad array of decomposition related enzymes. After fourteen days, medium extracts were analyzed (five technical replicates per biological replicate) for activity of a broad

spectrum of carbon decomposition related enzymes: i) cellobiohydrolase (cellulose degradation), ii) xylosidase (hemicellulose degradation), iii) glucosaminidase (chitin degradation), iv) oxidases and finally v) manganese- and vi) total peroxidases. The first three are hydrolytic, whereas the latter three are (per)oxidative enzymes. Finally, melanin content and hydrophobicity of some of the fungal isolates were measured in Lenaers *et al.* 2018 [23] and re-used here for analyzing the potential relationship with abiotic stress sensitivity.

### Quantification of sensitivity

We quantified growth rates by fitting a Verhulst (Lotka-Volterra based) growth model to the change in surface area through time (in the logarithmic space). This growth model is described by two parameters: intrinsic per capita growth rate ( $\mu_i$ ) and intraspecific competition ( $\alpha_{ii}$ ):

$$\frac{dX_i}{dt} = X_i(\mu_i + \alpha_{ii}X_i)$$

As  $\mu$  and  $\alpha$  are correlated, and showed similar responses, we only present the effects on  $\mu$ . Growth rate under control conditions ( $\mu$  at 23°C) was used as an explanatory variable relating with stress sensitivity. We quantified drought and temperature induced growth rate responses by calculating the relative change in  $\mu$ , expressed as the difference between  $\mu$  at the temperature stress levels (28 and 35°C) and  $\mu$  at the control temperature level (23°C), for both the drought (D) and control (C) water stress levels. Hence, four response variables describing different degrees of sensitivity were considered: i) mild temperature stress sensitivity (28C); ii) high temperature stress sensitivity (35C), iii) water and mild temperature stress sensitivity (28D) and iv) water and high temperature stress sensitivity (35D). The effect of water stress only can be assessed by comparing the responses for control and drought cases at different temperatures.

We used these data to test whether the ten fungal functional traits affected sensitivity to abiotic stress, using simple regression models separately for each trait. We expected melanin content, hydrophobicity, density, and (per)oxidative enzyme production to be positively related with stress tolerance; and color

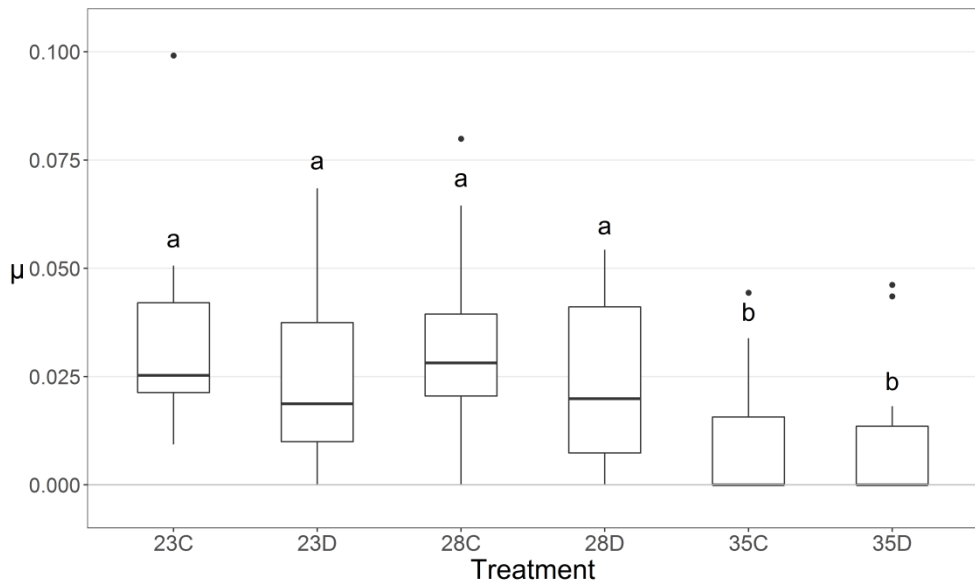
(considered as 'paleness'), growth rate and hydrolytic enzyme production to be negatively related with stress tolerance (Table 2). Each of ten traits was tested for a relationship with the four sensitivity response variables separately as well as combined into one average value depicting overall abiotic stress sensitivity. Additionally, we tested whether responses to temperature and water stress varied with species identity, at the order and division level (Table 1).

**Table 2:** Measured traits and their expected relationship with tolerance to temperature and water stress.

<b>Trait</b>	<b>Expected effect</b>
<b>Melanin content</b>	Positive
<b>Hydrophobicity</b>	Positive
<b>Density</b>	Positive
<b>(Per)oxidative enzyme production (3)</b>	Positive
<b>Color (paleness)</b>	Negative
<b>Growth rate</b>	Negative
<b>Hydrolytic enzyme production (3)</b>	Negative

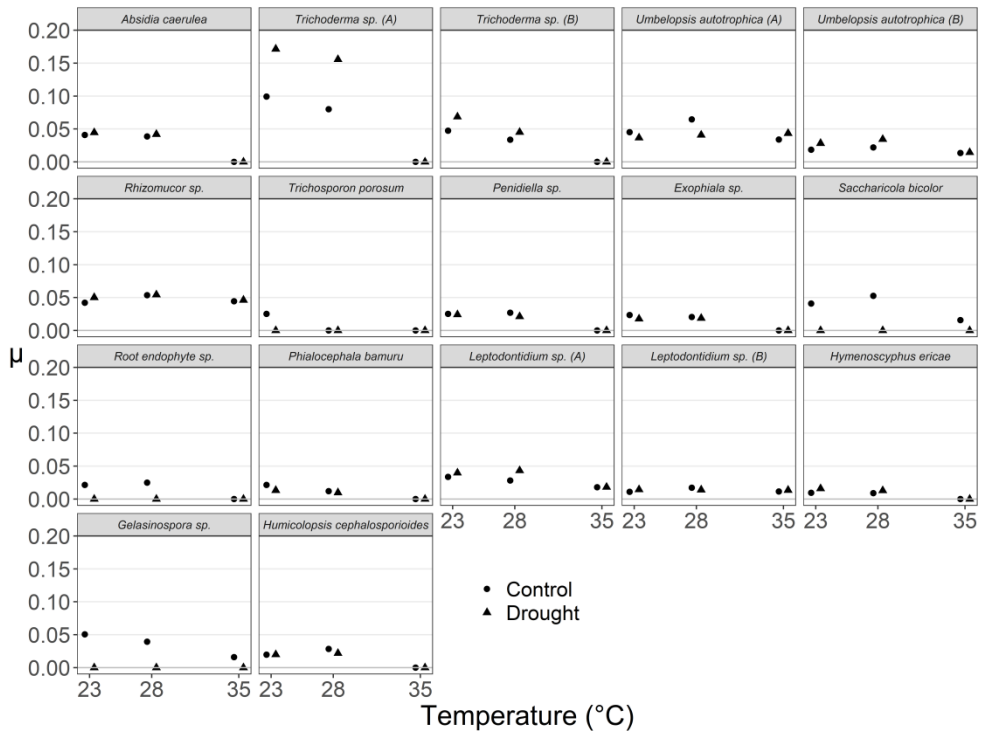
## Results

We found a significant effect of temperature, but not water stress on the growth rate of all taxa ( $n=17$ ). High ( $35^{\circ}\text{C}$ ) temperature stress, but not mild ( $28^{\circ}\text{C}$ ) temperature or water stress, significantly reduced growth rates ( $P < 0.001$ ) (Fig. 1).



**Figure 1:** Boxplots of the growth rates ( $\mu$ ) of all taxa for each treatment, showing a significant ( $p < 0.001$ ) decrease at  $35^{\circ}\text{C}$  (treatments 35C and 35D). Different letters indicate significant differences. Two datapoints were higher than 0.1 (visible in Fig. 2).

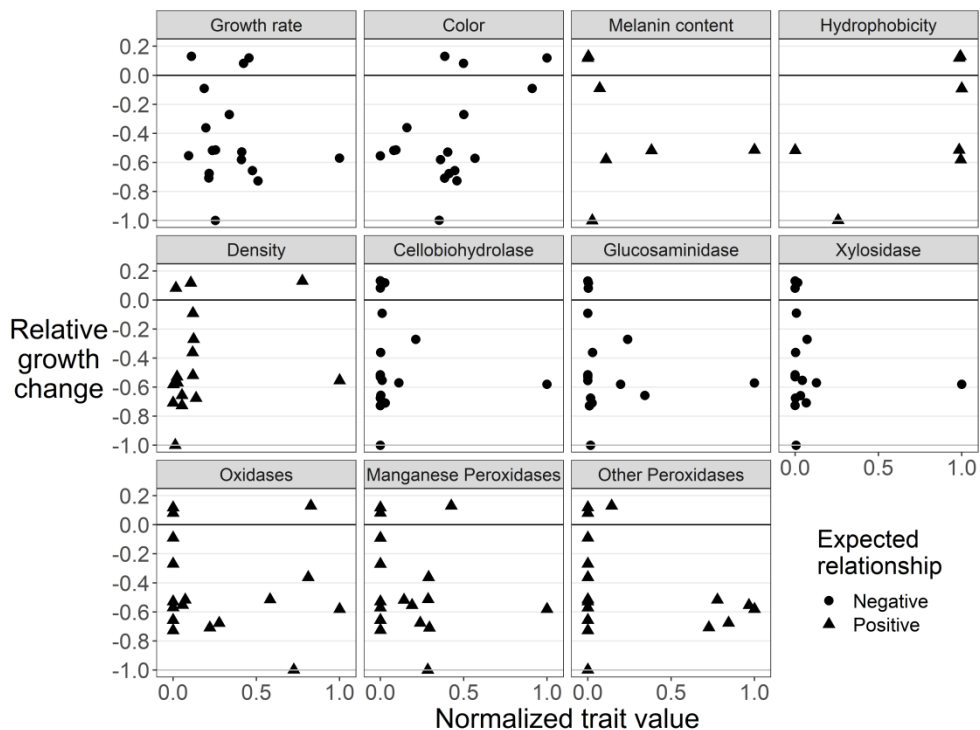
Despite the general effect of high temperature stress only, taxon specific responses to temperature and water stress were highly variable (Fig. 2; SI Fig. 1). More specifically, changes in growth rate among taxa under different levels of temperature and water stress varied from a 100% reduction to a 60% increase. For water as well as both degrees of temperature stress, some taxa grew faster, some grew slower and some were not affected.



**Figure 2:** Overview of the growth rates ( $\mu$ ) at each of six treatments for all seventeen taxa separately, showing the large variability in growth responses among taxa to temperature and water stress.

Despite the large variability in stress responses among taxa, we found no significant effect of any measured trait (Table 2) on abiotic stress sensitivity at any level (SI Fig. 1). Fig. 3 shows this lack of a relationship between all measured traits and overall stress sensitivity of all seventeen taxa. Color, as paleness, thereby showed a non-significant relationship with abiotic stress tolerance opposite to what was expected, as the pale *Umbelopsis autotrophica* taxa were most tolerant whereas (intermediately) dark taxa were most sensitive to abiotic stress (SI Fig. 2). Only taxonomy at the division level, not order, showed a significant effect on the overall response to abiotic stress, whereby Mucoromycetes are more tolerant than Ascomycetes (SI Fig. 3). However, this has to be considered carefully because of the biased distribution of taxa among groups.





**Figure 3:** Lack of relationship between overall stress response (average of four levels) and all continuous traits, normalized between 0 and 1 representing respectively minimal and/or maximal trait value among taxa.

## Discussion

### Stress responses

The growth rate of the seventeen fungal taxa was reduced significantly by high temperature stress (35°C), whereas mild temperature (28°C) had no effect on growth compared to the control for all taxa combined (i.e. 23°C) (Fig. 1). Additionally, for all taxa combined, there was no effect of water stress on growth at any temperature level, although we detected significant taxon-specific variation in sensitivity to water stress (Fig. 2 and SI Fig. 1). Hence, there is no effect over all taxa, but drought does have an important impact as there are highly varying differential responses. For example, water stress increased the growth rate of *Trichoderma sp.* (A) and (B), *Umbelopsis autotrophica* (B) and *Hymenoscyphus ericae*, and reduced or prevented growth in for example *Phialocephala bamuru* and *Gelasinospora sp.*, but had no effect on growth rates of *Absidia caerulea*, *Exophiala sp.* and *Penidiella sp.*. Hence, the expected overall reduction of fungal growth rates due to water stress was balanced out by an unexpected growth increase in some taxa. Also, the effect of mild increases in temperature (28°C compared to 23°C) showed variable effects on growth rates, with growth rate increases in for example both *Umbelopsis autotrophica* individuals and *Humicolopsis cephalosporioides*, growth rate reductions for *Phialocephala bamuru* and *Trichoderma sp.* (B) and no changes in growth in for example *Exophiala sp.* and *Penidiella sp.*. Finally, large increases in temperature (35°C compared to 23°C) reduced growth rates (up to 100%) in all taxa except for *Umbelopsis autotrophica* (A), *Rhizomucor sp.* and *Leptodontidium sp.*. We also detected intra-specific variation in stress responses, as *Umbelopsis autotrophica* (A) was relatively tolerant to high temperature stress, whilst the growth rate of isolate B was severely reduced at 35°C.

Hence, the observed large variability among fungal taxa in growth rates responses to temperature and water stress depicts a large variability in abiotic stress tolerances among taxa. Due to the lack of studies on abiotic stress sensitivities of these particular fungal taxa and the high functional variability of

closely related taxa, it is difficult to put these results into perspective. In general, the heathland soil fungal community can be considered relatively tolerant, as only a large increase in temperature up to 35°C significantly reduced growth rates in most taxa. These quantified optimal growth rates are of course much higher than those generally realized under different environmental conditions *in situ*.

Fungi were grown at 23°C, 28°C and 35°C, which were considered as control temperature, mild temperature stress and high temperature stress respectively. The optimal temperature for growth of fungi in temperate soils generally is around 25-30°C, with the maximum temperature for possible growth usually being around 10°C higher [112]. This explains why some taxa did not experience 28°C as (mild) stress, and most taxa did experience 35°C as stress. More specifically, at the research area in the National Park Hoge Kempen in Belgium, the mean temperature at 10cm depth of these poorly plant-covered sandy soils was 17°C during spring and summer of 2019, with occasional warming events up to 26.7°C [16]. Soil temperatures can be 10 to 30% higher at the surface than at 10cm depth [124], especially during the warmest periods [125]. Hence, the upper surface layer of these heathland soils, where most of the microbial activity occurs, can likely occasionally reach temperatures up to 35°C on sunny summer days. Thereby, these heathland soil fungi might thus have adapted a higher temperature optimum. Crowther and Bradford [126] illustrated the ability of soil saprotrophic fungi to rapidly acclimate to warming. Although we thus lack taxon-specific reference data, the observed unexpected growth rate increases of some taxa under elevated temperatures, even 35°C, might thus be caused by an adaptation towards tolerating higher temperature levels. The relatively harsh environmental conditions experienced in heathland soils might thus have caused a selective pressure towards a community of stress tolerant taxa. Alternatively, the increases in growth rate under mild temperature stress could be a hormesis response, a phenomenon which has been illustrated in plants [127], in which a stressor that has negative effects at high dosage (here 35°C), can induce positive effects at intermediate dosage (here 28°C).

In contrast to temperature, we did not apply water stress continuously throughout the experiment, but we rather transferred the fungus to an empty petri-dish for 72h prior to the start of growth at the different temperatures. As such, fungi experienced acute water stress, but also nutrient stress. However, we expect the latter to be of minor influence as trials had shown that all fungi showed none or non-significant growth decreases when growing on nitrogen depleted growth medium for several successive generations. After the 72h acute water stress period, moisture levels recovered during the 30 day temperature stress experimental period. Therefore, we measured resilience rather than resistance to water stress. At the research area, the median soil water content was only 3.7% during the same period, which was far below the observed 25 to 30% field capacity of these soils [16]. Therefore, in line with temperature stress, these heathland soil fungi might have adapted a strong resistance and resilience to water stress.

### **Relationship with functional traits**

We expected melanin content, hydrophobicity, density and (per)oxidative enzymes production to be positively related and growth rate, paleness and hydrolytic enzymes production to be negatively related with stress tolerance. But contrary to expectations, none of these functional traits explained variation in stress responses among taxa (Fig. 3). Only taxonomy, at the division level, showed a relationship with stress tolerance, whereby Mucoromycetes were more tolerant than Ascomycetes (SI Fig. 3). This is opposite to expectations, as these Mucoromycota generally are mold-type taxa with a fast-growing ruderal life-history strategy, and are therefore considered to be generally stress sensitive [128]. However, these results have to be considered with caution because of the biased distribution of the number of individuals among taxonomic groups (SI Fig. 3). Melanin content is known to be an important predictor of abiotic stress tolerance in fungi and color is considered an accurate proxy for melanin content [61]. However, in this experiment, neither melanin content nor color related to fungal abiotic stress tolerances (Fig. 3 and SI Fig. 2). Both strains of the palest taxon, *Umbelopsis autotrophica*, were most tolerant to temperature stress. Hence, this might indicate that for these heathland soil fungi, taxa that are not

melanized but stress tolerant, likely contain other protective compounds such as osmolytes [129] or heat-shock proteins [118], that we did not measure. The variation in abiotic stress tolerance among taxa might thus be caused by differences in other protective compounds than melanin. Additionally, we did not find a relationship between stress sensitivity and hydrophobicity, density and production of different types of enzymes. These key functional fungal traits have been raised as potentially directly or indirectly important in shaping stress sensitivities [77], [123]. But we did not find a relationship of any of the measured enzymes nor mycelial morphology with the tolerance of heathland soil fungi to warming and water stress. As we quantified the resilience rather than the resistance of fungi to water stress, we could have expected traits that are more important for recovery/resilience than for resistance, such as growth rate, to positively correlate with resilience to water stress (rather than negatively correlate with resistance to water stress). However, also growth rate did not shape the tolerance of fungi to water (nor temperature) stress in any direction.

### **Relevance**

Categorical groupings are limited in their capacity to differentiate between mechanisms shaping sensitivity because taxa within the same groups can display a wide range of trait values [118]. Hence, our use of continuous trait variables is an important strength. However, many of the traits we measured, such as hydrophobicity and hydrolytic enzymes, generally do not show much variation, but rather an 'all or nothing response', with many taxa showing low trait values and one or a few 'outliers' (Fig. 3). In line with the use of categorical groupings and despite data transformations, this biased trait distribution limits explanatory power, possibly partly accounting for the observed lack of relationship between functional traits and stress sensitivity. Although quantifying all these traits and tolerances for seventeen fungal species is a large effort, a sample size of seventeen is relatively low, contributing to the possibility that the statistical power might not always have been sufficient to show possible relationships. Additionally, fungi can be highly plastic, with spatiotemporal variation in morphology and physiology, which can obscure differences in life history strategies observed between taxa [117]. Hence, despite controlled

culturing and experimental set-ups, the observed lack of relationships between traits and stress response might also be partly explained by non-optimal conditions to quantify traits. Thus, the observed lack of a relationship between fungal traits and abiotic stress tolerance might be caused by the importance of other, non-measured traits, fungal plasticity and insufficient statistical power.

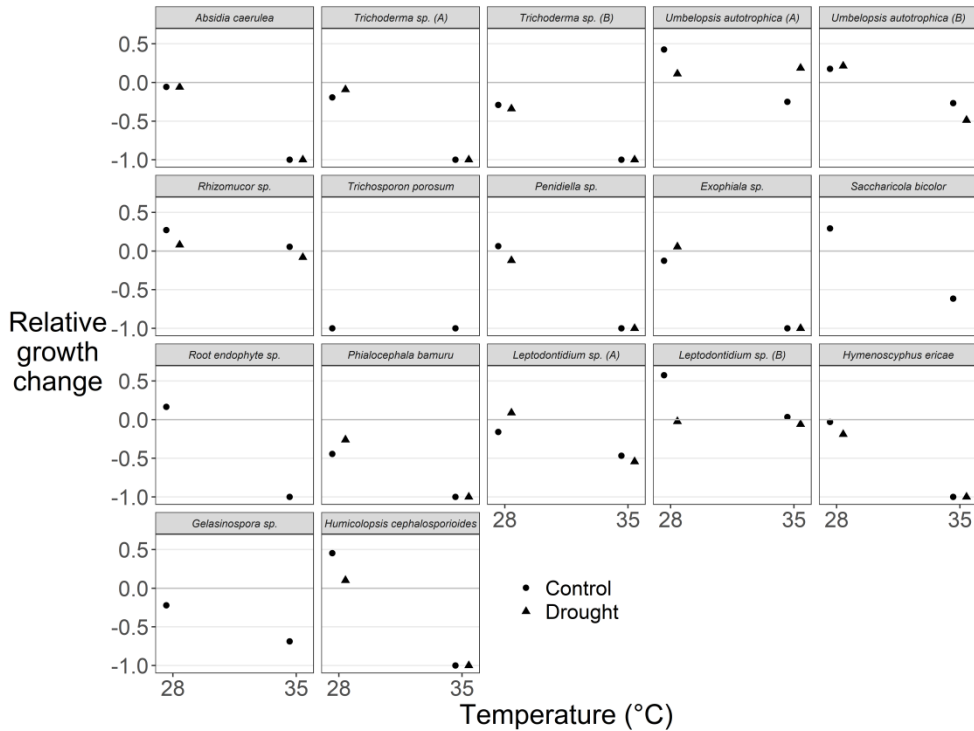
Several other adjustments to the design and set-up of this experiment would have improved the relevance of the gained results. A more natural way to have simulated water stress could be to expose fungi to dry sterile sandy soil rather than an empty petri-dish [130]. Additionally, rather than exposure to an acute water stress event, cycles of drying and rewetting better mimics realistic environmental conditions. However, this is difficult to apply in a set-up in petri-dishes and can be more easily applied in for example soil microcosms. Also, a broader temperature gradient with more points would have been useful to more accurately quantify taxon-specific responses to warming stress. Also, for optimal comparison and interpretation, traits values should have been assessed during the exposure to abiotic stress, or at least in similar set-ups. Finally, the effects of the abiotic stressors on surface area correlated with biomass, hence there were no changes in mycelial density that could have biased the conclusions made based on surface area of the fungi as a proxy for growth.

## Conclusions

Our results show that the heathland soil fungal community is generally tolerant to temperature and water stress, as only high temperatures (35°C) reduced overall growth rates. This general tolerance to abiotic stress might be caused by an adaptation to the relatively harsh edaphic conditions experienced in heathland soils, such as low water contents and high temperatures during summer. However, we detected large variability in responses to these abiotic stressors among taxa, with increases, decreases, and no changes in growth under each type and level of stress. This indicates that while the fungal community as a whole might not necessarily change in biomass, it is likely that abiotic stress will alter the composition and consequently the functioning of heathland soil fungal communities.

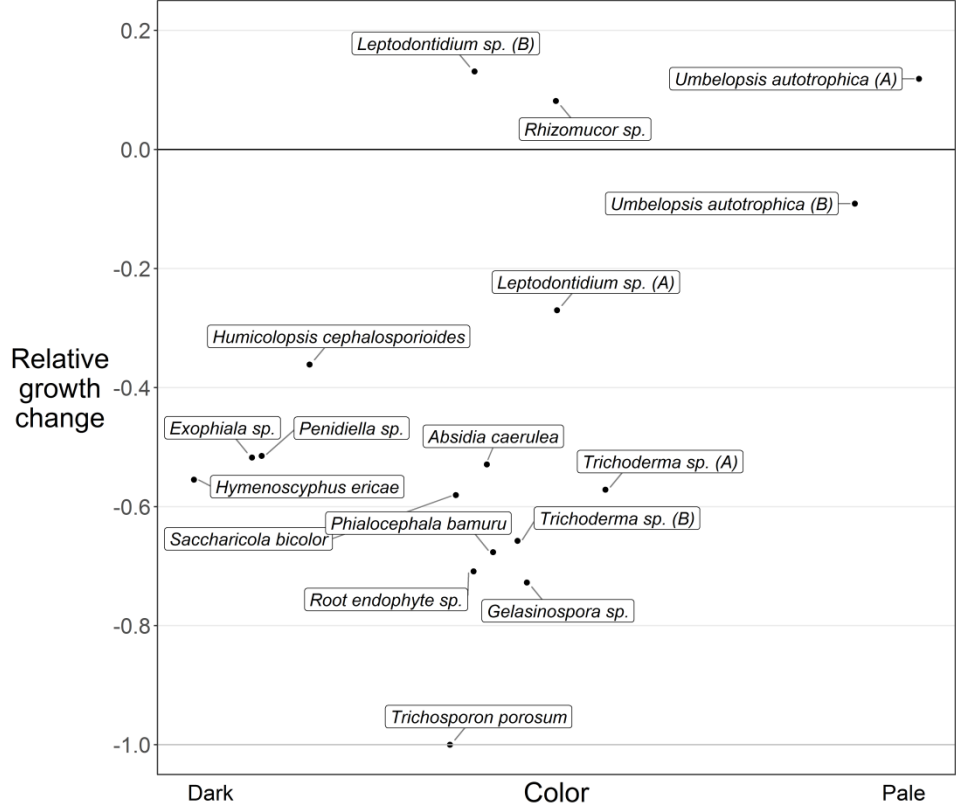
The lack of a relationship between several key functional traits and tolerance to temperature and water stress likely indicates that other traits than those measured in this study shape the sensitivity of heathland soil fungi to these abiotic stressors. The use of functional traits to describe taxa has been successfully used in plant and animal ecology, but for the desired shift in fungal ecology to likewise trait-based approaches, more empirical research on functional traits is thus needed to validate such frameworks. Therefore, we advocate for more elaborated studies that investigate which response traits shape the life history strategies of soil fungi, but also which effect traits further cause changes in ecosystem functioning [118]. Such an understanding would ultimately improve our predictions of how climate change will alter soil carbon sequestration through changes in soil fungal communities.

## Supplementary Information

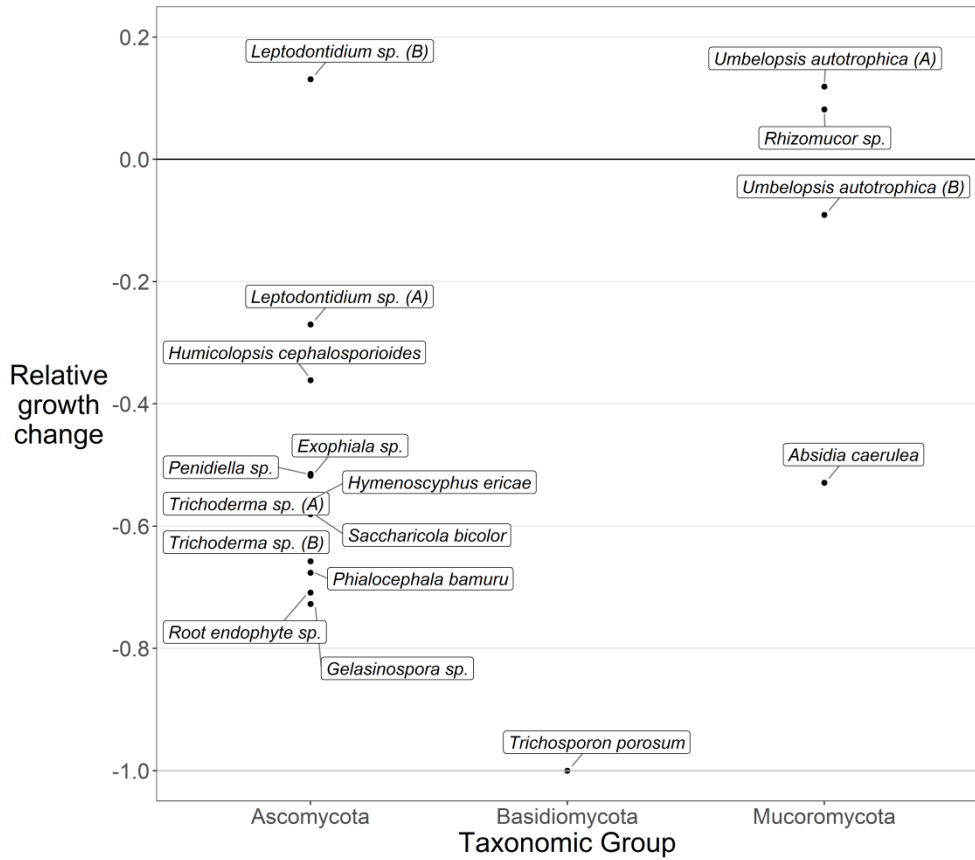


**Figure 1:** Overview of the relative change in growth rates of the four stress treatments over the control (23°C) treatments for all seventeen taxa separately, showing the large variability in stress responses. Changes vary from a 100% reduction (no growth) up to a 60% increase. Note that for some taxa, data is lacking for both drought cases, indicating lack of growth at the 23D treatment.





**Figure 2:** Relationship between fungal mycelial color and overall stress response (average of four levels).



**Figure 3:** Overall stress response of all seventeen taxa per taxonomic group.



# **Chapter 4**

## **Fungal pairwise interactions shift from positive to negative under warming stress**

Wouter Reyns, Francois Rineau, Camille Carpentier, Fons van der Plas, Richard D. Bardgett, Natalie Beenaerts and Frederik De Laender

WR, FR and FDL designed the experiment in consultation with all other authors. The experiment was performed by WR and analyzed by WR, FR, CC and FDL. WR wrote the main manuscript text, which was reviewed by all other authors.

## **Abstract**

The impact of climate change on soil processes such as carbon sequestration depends on how abiotic stressors such as drought and warming affect species interactions in key soil communities such as fungi. Therefore, we investigated how temperature stress and drought, as water stress, affect the capacity of soil fungi to grow in presence of a more abundant competitor, which we here define as biotic stress tolerance. In absence of abiotic stress, the competitor mostly affected growth positively, demonstrating facilitation among soil fungal species under benign conditions. In presence of temperature stress, either alone or combined with water stress, these positive effects became negative, which can have detrimental consequences for community composition and contradicts the stress-gradient hypothesis. We did not find biotic stress tolerance to be related to abiotic stress tolerance, nor to intrinsic growth rate. Hence, the shift from positive to negative interactions under abiotic stress is not predictable from the tolerance to abiotic stress or intrinsic growth of the focal species. Our results suggest that global change could potentially impact fungal communities in unpredictable ways.

## Introduction

The interactive effects of biotic and abiotic factors are key in determining the functional and ecological responses of microbial communities to climate change [131]. Specifically, understanding how species interactions change with environmental change is needed to anticipate consequences for ecosystem functions [132]. However, research on soil fungal interactions thus far is limited, hampering our understanding of the consequences of climate change for soil processes such as carbon sequestration. Most research so far investigated soil microbial communities through network analysis [133], [134], thereby focusing on species co-occurrences, without explicit quantification of interactions and thus underlying mechanisms. There is a need to complement such studies with pairwise interaction experiments, as pairwise interactions form the cornerstone of community dynamics [135].

So far, experiments have mainly assessed interaction type visually, and computed indices of dominance that assign numerical scores to each interaction type [80], [131], [136], [137]. Outcomes of such fungal interaction experiments were affected by the identity of the interacting species [136] as well as environmental conditions such as warming and water availability [131], [138], [139]. These approaches recognize the diversity of interaction types found back in fungi communities, including mutualism, parasitism, predation, and competition, whereby the latter is considered most common. Such interaction types often rely on the release of different types of exudates. For example, fungi release metabolites that enhance their ability to capture previously colonized substrates or to defend their own substrate base [140]. Hence, some studies investigated exudate production during pairwise interactions [141], as the production of secondary metabolites such as mycotoxins and organic acids could be a way in which they are able to spatially outcompete the opponent [142], [143], although the latter could also serve as an energy source to other fungi.

While available interaction studies have documented the potential ways in which fungi can interact, they cannot replace more quantitative approaches that measure species interactions, as well as how these respond to environmental

## Chapter 4

factors relevant to global change. Here, we therefore investigate how the growth of fungi isolated from a heathland soil is affected by the presence of another fungal species. Additionally, we test how this interaction is affected by increased temperatures and drought, two main climate change-related environmental stressors. The stress gradient hypothesis (SGH), often observed in plant ecology experiments [144], predicts a shift from more competitive interactions under benign conditions to more facilitative interactions under stress conditions.

In the previous chapter, despite large variations among taxa, we found no significant effect of mild warming and water stress (drought) on overall growth rates, but a large negative effect of high warming stress on overall growth rates (Chapter 3 Fig. 1). First, in line with the SGH, we therefore expect a shift from more negative (competitive) interactions under control, drought and mild warming (generally no stress perceived) to more positive (facilitative) interactions at 35°C (generally perceived as high temperature stress). By explicitly considering parameters that define the life-history of the interacting species, we are able to test specific predictions relevant to the SGH [145], [146]. Secondly, we therefore additionally assessed whether the changes in fungal growth rates due to both biotic stress (presence of another taxon) and abiotic stress (warming and drought) are influenced by the interacting species' intrinsic growth rate (at control) and tolerance to these abiotic stressors. We expect the intrinsically faster the focal species is compared to the resident, the higher its capacity to successfully grow in presence of a resident, and hence the more tolerant to biotic stress. We expect this positive relationship to be also present for abiotic stress tolerance, with the more tolerant to abiotic stress the focal is compared to the resident, the higher its capacity to successfully grow in presence of a resident under high abiotic stress (35°C).

## Materials and Methods

### Assigning treatment specific pairwise combinations

In Chapter 3 of the thesis, we exposed fungi in a full factorial design to i) increases in temperature (23°C as a control, 28°C as mild warming stress and 35°C as high warming stress) and ii) presence (D) vs. absence (C) of water stress (drought), leading to a total of six treatments (23C, 23D, 28C, 28D, 35C 35D). As we expect fungal growth rates in presence of a resident to be affected by the difference in abiotic stress sensitivity and intrinsic growth rate between the two competing species, we used these two variables to select treatment specific species pairs. More specifically, based on the monocultural stress experiment in Chapter 3 of the thesis, we calculated the difference in logarithmic control growth rate between species A and B ( $\log(\mu_{A\_23C}) - \log(\mu_{B\_23C})$ ) and the difference in logarithmic stress sensitivity (for example for the 28C treatment:  $\log(\mu_{A\_28C}/\mu_{A\_23C}) - \log(\mu_{B\_28C}/\mu_{B\_23C})$ ). This for all possible taxon combinations for all abiotic stress treatments considered (28C, 28D, 35C and 35D). In total, thirteen taxa were used (Table 1), whereby *Penidiella sp.*, *Exophiala sp.*, *Trichosporon porosum* and one *Leptodontidium sp.* taxon from the monocultural experiments in the previous chapter were not considered because they did not grow under the abiotic stress conditions or because of practical limitations. In order to have a standardized distribution of variation in the two variables considered, we selected species pairs within a range of 100% relative difference in intrinsic growth rate (y-axis) and 50% relative difference in stress sensitivity (x-axis) (Fig. 1). For each of four abiotic stress treatments, we randomly selected twelve taxon pairs, three within each of four quadrants, with higher or lower relative difference in abiotic stress sensitivity and higher or lower relative difference in intrinsic growth rate, demarcated within the aforementioned ranges. The observed negative correlation between both variables can be attributed to the relationship present between both considered parameters.



## **Experimental set-up and design**

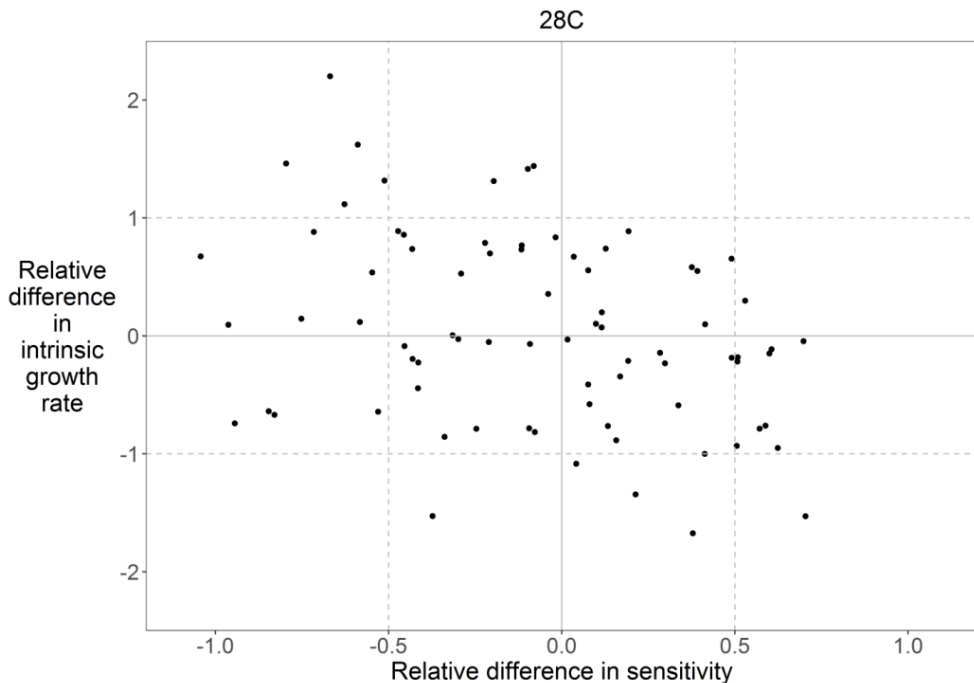
The experimental set-up and design was the same as the monocultural stress experiment described in Chapter 3. Here, one of either taxa, called the resident, was grown as a monoculture initially. But additionally, when the resident fungus reached approximately 50% of its carrying capacity (based on the monocultural growth experiment), we introduced the focal species as a small (three by three mm) mycelial plug. For all of the twelve treatment-specific taxon pairs, we performed invasion experiments in both directions (each taxon acted as focal as well as a resident). Additionally, treatment-specific bidirectional interaction experiments were performed for the particular stress treatment considered as well as its respective control temperature treatment (23C or 23D).

## **Quantification of the effect of interaction**

The effect of the resident on the growth of the focal taxon was quantified by comparing the growth rate of the focal taxon with its growth rate as a monoculture. More specifically, we calculated the logarithm of the ratio of its growth rate as a focal in presence of a resident over its growth rate under monocultural conditions under the same treatment (for example for the 28C treatment:  $\log(\mu_{28C\_withresident}/\mu_{28C\_asmonoculture})$ ). Hence, positive values indicate an increase in growth rate due to presence of a resident taxon (tolerant to biotic stress), whereas negative values indicate a decreased growth due to the presence of a resident fungal taxon (sensitive to biotic stress). Next to testing the effects of increases in temperature separately for control and drought cases (the aforementioned four abiotic stress treatments), the combined effects of temperature and drought were also tested by comparing all treatments, including drought, to the 23C reference (giving five abiotic stress treatments: 23D, 28C, 28D, 35C, 35D; whereby the drought treatments differ from the original (four treatment) approach).

**Table 1:** Experimental fungal taxa and the order and division (taxonomic group) they belong to.

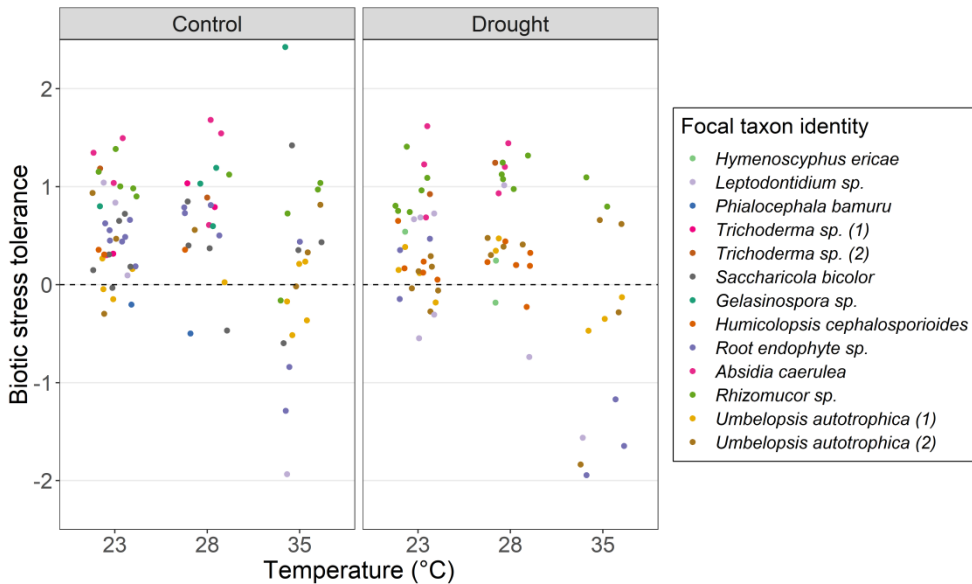
Taxon	Order	Division
<i>Hymenoscyphus ericae</i>	Helotiales	Ascomycota
<i>Leptodontidium sp.</i>	Helotiales	Ascomycota
<i>Phialocephala bamuru</i>	Helotiales	Ascomycota
<i>Trichoderma sp.</i> (2)	Hypocreales	Ascomycota
<i>Saccharicola bicolor</i>	Pleosporales	Ascomycota
<i>Gelasinospora sp.</i>	Sordariales	Ascomycota
<i>Humicolopsis cephalosporioides</i>	<i>incertae sedis</i>	Ascomycota
Root endophyte <i>sp.</i>	/	Ascomycota
<i>Absidia caerulea</i>	Mucorales	Mucoromycota
<i>Rhizomucor sp.</i>	Mucorales	Mucoromycota
<i>Umbelopsis autotrophica</i> (2)	Mucorales	Mucoromycota

**Figure 1:** Overview of all possible taxon pairs for the 28C treatment. We randomly selected twelve pairs within the depicted frame of 50% relative difference in sensitivity to abiotic stress and 100% relative difference in intrinsic (control) growth rate (demarcated in dotted lines), three for each of four quadrants.

## Results

### Pairwise interactions under abiotic stress

In order to investigate how climate change (abiotic stress) affects the capacity of soil fungi to grow in presence of a more abundant competitor (biotic stress tolerance), we used a linear model predicting biotic tolerance based on the separate effects of temperature and water stress. The model accounts for mixed effects (combination of the identity of focal and resident taxa) and a greater variance of the response variable at 35°C. We found a high tolerance to biotic stress under control temperature conditions (treatments 23C and 23D) (Fig. 2), as there is a significant positive increase in growth when a fungus grows in presence of another taxon compared to its monocultural growth. This high biotic stress tolerance is not affected by mild temperature stress (28°C). However, under high temperature stress (35°C), this generally positive effect becomes more variable and negative, with a neutral average. There is no effect of water stress on biotic stress tolerance at any temperature level. These results are the same when only considering those taxa that grow in all treatments (SI Fig. 1), indicating that these observed relationships between climate change induced abiotic stress and growth changes under biotic stress (pairwise interactions) are not biased by the lack of growth of certain taxa under certain abiotic stress conditions.

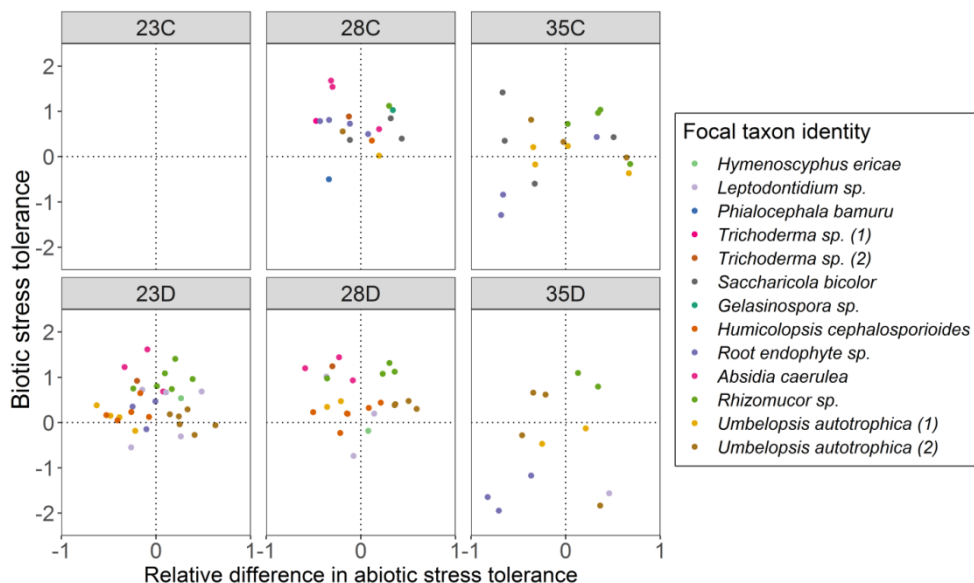


**Figure 2:** Biotic stress tolerance, expressed as relative change in growth rate due to presence of a competing resident taxon (pairwise interaction), for the six different water and temperature stress treatments considered. Resident presence generally significantly increases focal growth rates under control temperature and mild warming, whereas there is no significant change, but more variation including negative effects, under high temperature stress (35°C). Data are jittered per treatment to improve visualization.

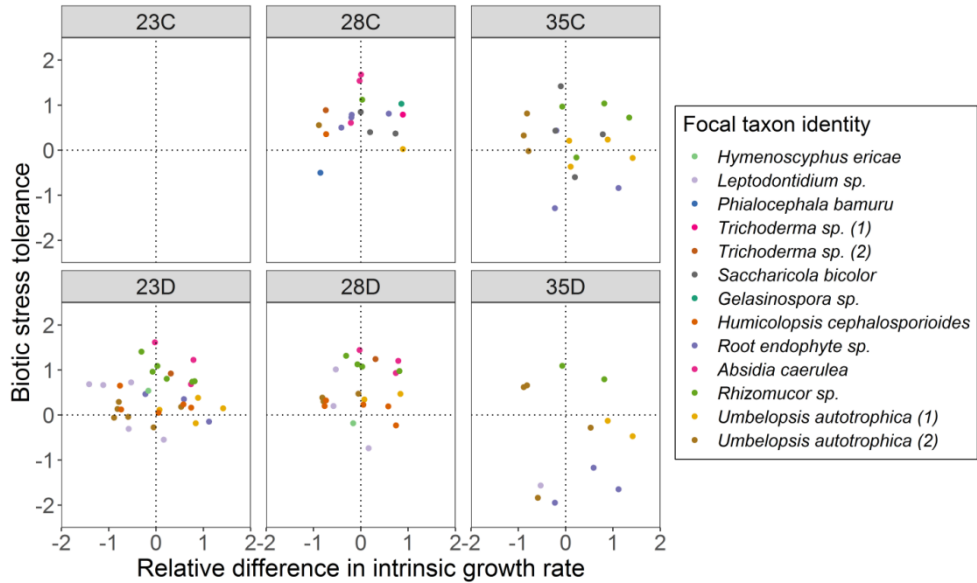
The growth response to a resident varied a lot among focal taxa (Fig. 2). For example, both *Umbelopsis autotrophica* isolates showed neutral responses, consistent among different treatments, whereas *Rhizomucor sp.* and *Abisidia caerulea* benefitted from presence of a resident taxon, as they showed consistent positive growth effects. Responses were less consistent within and among treatments for other taxa, whereby several taxa have, due to the randomized pairwise selection process, too few cases to make robust conclusions. *Leptodontidium sp.* and *root endophyte sp.* are the two most biotic stress sensitive taxa under high temperature stress, as these taxa showed large negative changes in growth rate due to presence of a resident. Despite some negative outliers at 35°C, *Umbelopsis autotrophica*, *Rhizomucor sp.* and *Abisidia caerulea* also had consistent positive growth effects on the focal taxon when acting as a resident (SI Fig. 2).

### The influence of tolerance to abiotic stress and intrinsic growth rate

In order to investigate whether the difference in abiotic stress tolerance and intrinsic growth rate between the two interacting taxa affects biotic stress tolerance of the focal taxon, we used a linear model predicting biotic stress tolerance based on the interaction between the abiotic stress treatment and the difference in abiotic stress tolerance or intrinsic growth rate respectively. Accordingly, the model accounts for mixed effects and a greater variance of the response variable at 35°C. In contrary to expectations, we did not find an effect of the difference in abiotic stress tolerance (Fig. 3) nor intrinsic growth rate (Fig. 4) between focal and resident taxa on biotic tolerance of the focal taxon at any of five considered abiotic stress treatments. But since the resident is already established upon invasion, the abiotic stress tolerance is possibly not very important. Therefore, we additionally investigated whether the abiotic tolerance and intrinsic growth rate of the focal taxon only affected its biotic tolerance. But despite indications of a positive relationship at 35D for abiotic stress tolerance and 23D, 28D and 35D for intrinsic growth rate, they were not significant (SI Fig. 3 and 4).



**Figure 3:** Relationship between the relative difference in abiotic stress tolerance between invader and resident and the invader's biotic stress tolerance, showing the lack of a significant relationship in all five treatments.



**Figure 4:** Relationship between the relative difference in intrinsic growth rate between invader and resident and the invader's biotic stress tolerance, showing the lack of a significant relationship in all five treatments.

## Discussion

### Pairwise interactions under abiotic stress

The unexpected general increased growth under control conditions of the invading fungus due to presence of a resident fungus (Fig. 1) can be interpreted as a facilitation effect. A possible underlying mechanism of facilitation might be the production of secondary metabolites by the established resident, such as a variety of organic acids [147], [148], which could improve carbon availability and thereby prime the growth of the invading fungus. However, as such exudates are lower in quantity and likely also in quality than the glucose provided in the growth medium, this is likely not the main driver of the observed positive growth effect. This increase in growth rate, potentially in order to spatially outcompete the opponent, could be compensated by less dense growth. However, as we practically could not measure the biomass of the two fungal taxa separately, we cannot judge whether a larger surface area is compensated by less dense growth (see further). But indeed, the taxa that show the most consistent and highest increases in growth due to the presence of a resident are *Rhizomucor sp.* and *Abisidia caerulea* (Fig. 2), both Mucoromycetes with a ruderal life history strategy, characterized by high growth, dispersal and colonization rates, and also relatively low mycelial densities [Reyns unpublished data]. The growth rate of these taxa is not only increased by the presence of a resident, but they also consistently increase the growth rate of any focal taxon when acting as a resident themselves (SI Fig. 2). Although we did not visually observe exudate production, the latter can potentially be caused because these ruderal-type taxa typically produce high amounts of exudate organic acids [141], that thus might serve as an energy source. The growth of the other Mucoromycete taxon, *Umbelopsis autotrophica*, which has been tested in duplicate, is not positively affected by biotic stress, but this taxon also positively affects the growth of the focal when present as a resident. Ruderal type fungi are often mycotoxicogenic and thereby assumed to produce secondary metabolites as part of maintaining a competitive edge over other taxa [142]. Our results indicate that these ruderal Mucoromycete fungi play an important

role in soil fungal community succession by promoting rather than inhibiting growth of interacting fungal taxa.

The positive effect of the presence of a resident on the growth of the focal taxon was not affected by mild warming or by water stress at any temperature level (Fig. 1). However, fungal interactions became more negative at high temperature stress (35°C) compared to control conditions. Hence, these results contradict the stress-gradient hypothesis (SGH) apparent in plant ecological experiments, which, based upon the plant strategy theory of Grime [121], predicts a shift from competitive to positive (facilitative) interactions as abiotic stress increases [144]. However, recent empirical research in plant ecology has also contradicted this hypothesis [149], [150], whereby factors such as the nature of the considered stressor and variation among species influence the outcome of species interactions. Also, abiotic stress could mostly determine what species compete for, rather than how strongly they compete, which might be an additional reason why our observed patterns of interactions under abiotic stress deviate from the proposed SGH. For fungi, substantial empirical investigations of the combined effect of biotic and abiotic stress are limited. Duarte *et al.* [139] however found a similar, SGH contradicting response for aquatic fungi under temperature stress, with pairwise interactions leading to an increase in growth under optimal temperature conditions, but a decrease under warming stress. Hence, in line with more recent findings in plant ecology, also for fungi, and microbial ecology in general, species interactions under abiotic stress might be more complex than proposed in the SGH, whereby a consideration of the species characteristics and nature of the stressor might greatly refine specific predictions [146].

The unidentified root endophyte taxon and *Leptodontidium sp.* are most sensitive to biotic stress under high temperature stress (Fig. 3), indicating that these stressors can synergistically negatively affect fungal growth. These negative effects are potentially mediated by the elevated energetic demands associated with competitive interactions and enzymatic production [151]. This *root endophyte sp.* taxon visually produced most extracellular metabolites of all taxa. As these exudates were produced under all treatments during the



interaction experiment, but not during monocultural growth, we can assume these are biotic but not abiotic stress related, and it therefore does not explain the synergistic effect of warming on biotic stress. Such volatiles produced under fungal interactions can be highly variable in type [141] and thus inhibitory effect [140]. Interestingly, these interaction-induced exudates had no effect on the growth of the competing focal taxon when it acted as a resident itself, as these showed small positive or neutral effects, similar to other taxa acting as a resident (SI Fig. 2). Therefore, the production of these exudates is likely a response to perceiving biotic stress rather than a strategy to outcompete the other fungus (antagonism). Whether the observed negative growth changes are caused by competition for nutrients or space and/or by antagonism [152] remains speculation, as we did not measure changes in nutrient status of the petri-dish/fungi nor did we measure exudate production. Volatile identification and quantification can be achieved by ('time of flight'-based) GC-MS methods [153].

### **The influence of tolerance to abiotic stress and intrinsic growth rate**

We expected fungi that are more tolerant to abiotic stress to be more tolerant to biotic stress under abiotic stress. However, we did not find a relationship between biotic and abiotic stress tolerance at any abiotic stress treatment (Fig. 3 and SI Fig. 3). Accordingly, we expected, but did not find, a positive relationship between intrinsic growth rate and tolerance to biotic stress (Fig. 4 and SI Fig. 4). These intuitive hypotheses were opposite to what could be expected considering the general trade-off between competitive ability and intrinsic growth rate following the CSR framework [142] and the observation by Kuyper and Verschoor [154], who found that slower growing taxa were less negatively affected by the presence of another taxon compared to faster growing taxa. The same applies to the CSR-hypothesized positive relationship between biotic stress tolerance and abiotic stress tolerance. However, we thus found neither a positive nor a negative relationship between both intrinsic growth rate and abiotic stress tolerance with biotic stress tolerance. This might be caused by the dominance of facilitative rather than competitive interactions among our heathland soil fungi, especially under benign conditions, suggesting

that our quantified biotic stress tolerance is not an explicit measure of competitive ability under the CSR framework. Also, the rationale behind CSR-framework does not seem to fit our observations of for this heathland soil fungal community. Thus, other factors than those considered here should play a role in shaping interactions between heathland soil fungi.

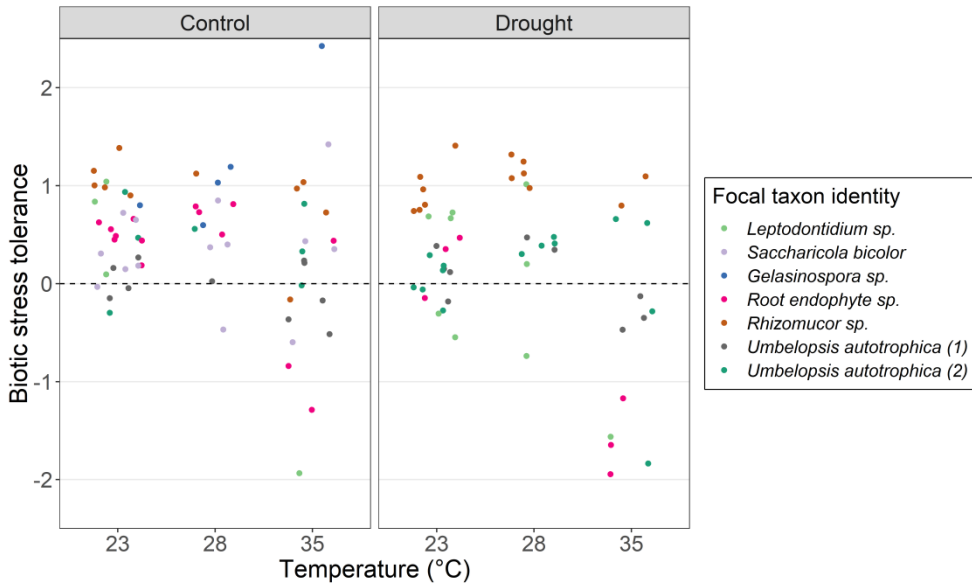
## Relevance

Several adjustments to the design and set-up of this experiment would have improved the relevance of the gained results. For example, as previously mentioned, we were not able to quantify the biomass of the interacting species separately. Therefore, we were not able to test whether changes in surface area correlated with changes in biomass, which we did observe under abiotic stress in Chapter 3. We were thus not able to test whether fungi escaped biotic stress by changing growth form (mycelial density). Also, in the artificial petri-dishes, there is no presence of symbiotic ericoid plants, which might therefore induce a disadvantage for ERM relative to saprotrophs when competing compared to real *in situ* conditions. Additionally, the competitive interactions experienced with other fungi likely are relatively limited *in situ*, especially since they are believed to have a less well-developed extraradical mycelial phase than ECM and AMF [155]. Finally, we considered 23°C as the optimal temperature for fungal growth and hence the lowest point of our temperature range. But, the mean *in situ* experienced temperature at 10cm depth at the research area in the National Park Hoge Kempen in Belgium was only 17°C during spring and summer of 2019. Hence, the considered temperature gradient might not have been extreme enough to relate to the SGH. As heathland soils are dry environments with occasional high soil surface temperatures during summer [16], these fungal communities likely are more adapted to these abiotic stressors than in other ecosystems such as grasslands and forests. Therefore, extrapolation to other fungal systems has to be considered carefully.

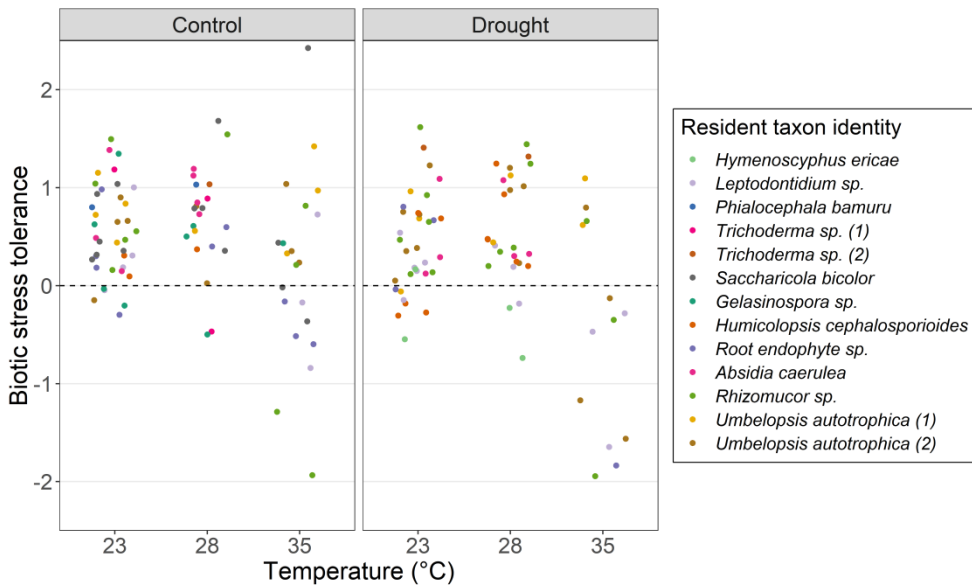
## Conclusions

Heathland soil fungi generally show positive growth responses to biotic interactions. These facilitative interaction effects might be mediated by the production of unidentified growth promoting exudates such as organic acids. However, under high temperature stress (35°C), the competing fungus had negative growth effects on the focal taxon, indicating that these stressors have a synergistic effect. Hence, interactions between fungi shift from positive (facilitative) to negative (competitive) under warming stress, opposite to predictions of the stress-gradient hypothesis (SGH). As abiotic stress tolerance and intrinsic growth rate showed no relationship with biotic stress tolerance, several other, unidentified mechanisms, should drive heathland soil fungal interactions. Our results suggest that climate change could potentially affect fungal community succession and thus carbon sequestration in unpredictable ways. In order to better assess the effects of climate change on soil processes such as carbon sequestration, we advocate for similar experiments investigating additional potential mechanistic drivers of climate change induced changes in fungal interactions.

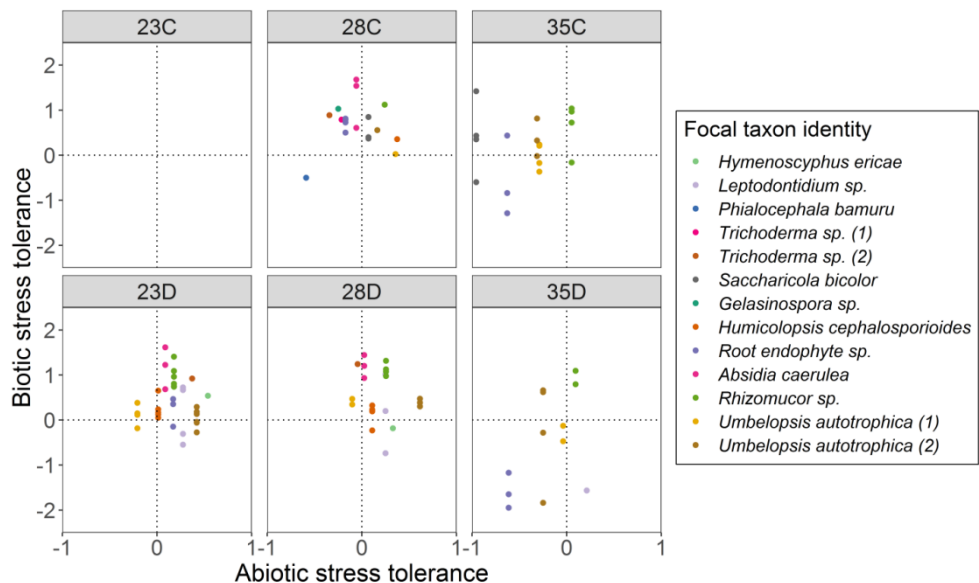
## Supplementary Information



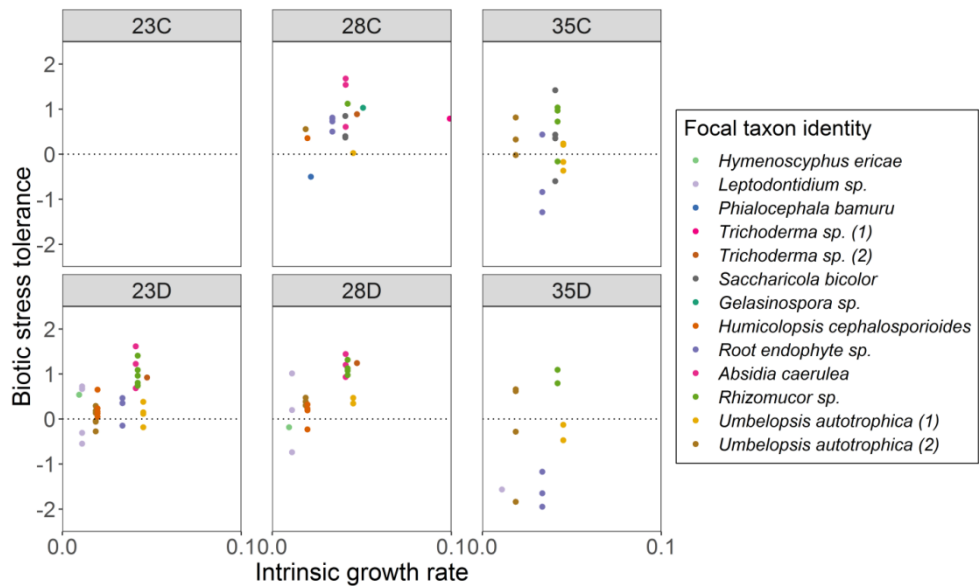
**Figure 1:** Biotic stress tolerance, expressed as relative change in growth rate due to presence of a competing resident taxon (pairwise interaction), for the six different treatments considered and only those fungal taxa that grow under all treatments.



**Figure 2:** Biotic stress tolerance for the thirteen experimental taxa as a resident.



**Figure 3:** Relationship between abiotic and biotic stress tolerance (relative change), showing a lack of a significant relationship in all five stress treatments.

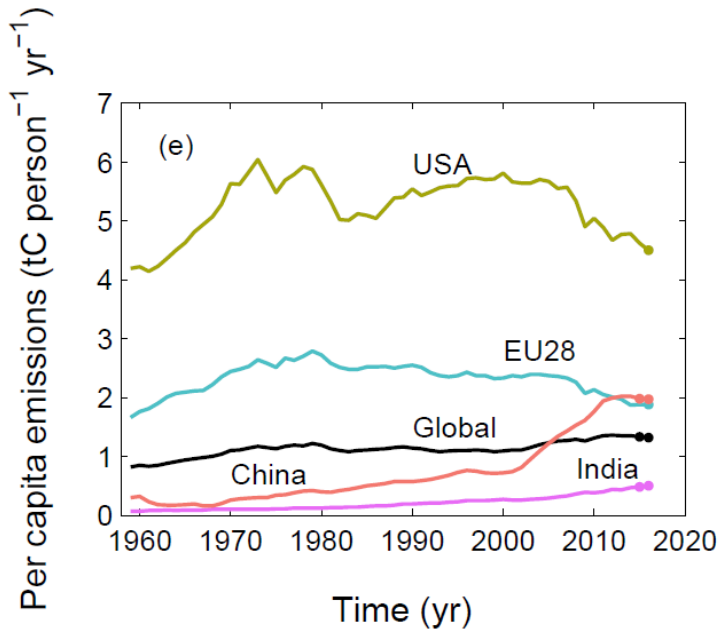


**Figure 4:** Relationship between intrinsic growth rate and biotic stress tolerance (relative change), showing a lack of a significant relationship in all five stress treatments.

## **General Discussion**

## **Science to mitigate climate change: investigating heathland soil fungal functioning**

The 2016 Paris Agreement to reduce greenhouse gas emissions has been signed by almost all nations in order to combat human-induced climate change [156]. However, most nations fail to achieve these goals, and as these goals are not binding, failing nations are not getting sanctioned. Additionally, the United States of America, which is by far the most carbon polluting nation considering per capita contribution (Fig. 1), withdrew from the agreement under the presidency of Donald J. Trump since January 2017. Another country with major global impact, Brazil, is also moving away from climate action and from fulfilling its commitments under the Paris Agreement, since the start of the presidency of Jair Bolsonaro in January 2019. Hence, despite worldwide increases in floods, droughts and other extreme climatic events that affect many countries globally, also indirectly through increases in refugees, the positive intentions from the Paris Agreement are halted due to the lack of sufficient measures undertaken by governments of non-believing or non-willing nations. Also in Belgium, the limited local governmental action has led to protests motivated by the idea that we are not doing enough to 'save our planet', leading to political and societal tension.



**Figure 1:** Per capita carbon emissions (tons of C per person per year), for the world's most emitting nations (with the 28 European Union nations combined), from le Quéré *et al.* 2018 [2].

In climate change science, we try to understand current and project future effects of human-induced climate changes on ecosystem functions, in order to seek opportunities to mitigate these effects. These opportunities could then be implemented in society through policymaking. Experimentally *in situ* observed changes in soil carbon contents under climate change simulations are very variable and the underlying mechanisms are poorly understood. We investigated this issue using heathland soils as a study system, which are dominated by fungi. Therefore, in this PhD, we aimed to better understand how heathland soil fungi are affected by climate change, as changes in soil fungal functioning to a large extent drive the observed changes in soil carbon sequestration. Part of the observed variability might be caused by the interplay between different climate change-induced abiotic stressors [157], advocating the importance to study the effect of different stressors simultaneously, as it can lead to synergistic, antagonistic or additive effects. Therefore, we investigated the combined effects of the two most important environmental change drivers affecting terrestrial ecosystem in Western Europe, warming (temperature stress) and drought



## General Discussion

(water stress) [3]. An increased awareness of climate change-induced changes in fungal functioning will eventually enhance predictability of the role that soils could play in climate change mitigation under future climate change.

First, we reviewed the information available in the literature regarding soil food web functioning in heathlands and how this is affected by climate change. Using a simple mathematical model, we quantified which knowledge gaps are most important to unravel in order to enhance predictive capacity (**Chapter 1**). We found that species interactions and especially stress tolerances are of major importance. Second, in order to use as many of the local heathland soil fungi as possible in laboratory experiments, we used several isolation methods and growth media to increase soil fungal isolation efficiency (**Chapter 2**). We found that all four tested isolation methods, which have largely varying methodologies, showed high taxon specificity and complementarity. However, the use of different growth media did not improve cultivation efficiency. By using these various isolation methods combined with long incubation times, we were able to isolate a relatively diverse fungal community. Third, based on the outcome of the quantitative literature review in Chapter 1, our specific subsequent research goals were twofold: i) characterize heathland soil fungal taxa regarding their sensitivity towards temperature and water stress (**Chapter 3**) and ii) investigate how interactions between fungi are affected by these abiotic stressors (**Chapter 4**). We are not aware of other experiments that provide such basic empirical knowledge of a whole soil fungal community at the taxon level. However, this is an important task in order to better understand soil fungal functioning at the level of the individual, before integration into predictive models.

## **The heathland soil fungal community is generally well-adapted to temperature and water stress, but fungal tolerance is unpredictable from their traits**

We found that the heathland soil fungal community was relatively tolerant and thus well adapted to abiotic stress, with an overall growth reduction only under high warming stress (35°C towards 23°C). However, interspecific variability in growth responses was high for both temperature and water stress, as some taxa grew slower, some were not affected and some grew faster, even under high temperature stress. Fungi thriving in heathland soils might be better adapted to these abiotic stressors compared to other systems where these species occur, because heathland soils are characterized by harsh edaphic conditions, with a very poor nutrient status, low pH, poor or very free water drainage, and high or low temperatures [158]. Also at the research area in the National Park Hoge Kempen in Belgium, high summer temperatures can occasionally be reached and a low median water contents was observed [16]. Therefore, these heathland soil fungi might be well adapted to warming and water stress. Regarding the latter, this is in line with some trials we conducted in small microcosm experiments, where we saw an unexpected negative relationship between CO<sub>2</sub> production (proxy for growth) and water content (ranging from 3 to 30%), for the taxon *Humicolopsis cephalosporioides* [Reyns unpublished data].

Several traits that were expected to shape tolerance to temperature and water stress, such as melanin content, did not show a relationship with sensitivity to these abiotic stressors. This questions the relevance of trait-based approaches, or at least the importance of those traits measured here, to predict community dynamics under environmental change. The observed variation in abiotic stress tolerance among taxa might thus be caused by other physiological and morphological functional traits than those that we measured. There was no significant correlation between sensitivity to both abiotic stressors, since taxa that were highly sensitive to drought were not necessarily highly sensitive to warming and vice versa. This might indicate that the functional traits that shape sensitivity to temperature stress might be different from those shaping

## General Discussion

sensitivity to water stress. This could explain the lack of a significant relationship between both water and temperature stress with melanin, as this pigment serves as a protector to both abiotic stressors simultaneously [61]. Melanin could thus play a less important role in the stress tolerance of this heathland fungal community than other, stressor-specific traits. For example, the observed interspecific variation in water stress tolerance of these heathland soil fungi might be mainly caused by differences in for example the degree of production of certain osmolytes such as trehalose [77], [118], which was not quantified. Hence, for the desired shift in fungal ecology to trait-based approaches [117], further empirical validation of the existing conceptual frameworks is needed.

Several taxa of the fungi that were isolated are poorly known as they are specifically found in acidic nutrient-poor soils. Therefore, available literature knowledge on their characteristics regarding stress tolerance is very scarce. In ecological studies, scientists therefore often infer traits from large databases, such as the TRY plant database for plant ecology [159]. However, the large functional variability in closely related fungi, even intraspecifically [160], [161], which was also the case for our two *Umbelopsis autotrophica* strains, shows the need to measure traits from locally occurring fungi, rather than extrapolate information from available literature. In fauna [86] and especially plant [162]–[165] ecology, it has been extensively illustrated that traits can be plastic and highly variable and it is therefore necessary to specifically address the relative importance of intra- and interspecific variability in communities in order to correctly use trait-based approaches and better understand community assembly. Additionally, de Bello *et al.* proposed methods to account for intraspecific variability when measuring functional diversity [166], as intraspecific variability can be a key driver for the maintenance of biodiversity under environmental change [167]. However, in fungal ecology, the importance of intraspecific diversity has been poorly studied [168], [169].

## **Positive pairwise interactions under benign conditions become negative under temperature stress, are not shaped by abiotic stress tolerance nor intrinsic growth rates**

This PhD illustrated that independent of their abiotic stress tolerance and intrinsic growth rate, all heathland soil fungi were tolerant to biotic stress, whereby their growth rate in presence of a more abundant resident was higher than in monoculture. Under high temperature stress however, for which most taxa were sensitive when grown in monoculture, these positive effects of biotic stress shifted towards neutral and negative effects compared to monoculture growth. However, also for the 35°C treatments, there was no significant interaction between abiotic and biotic stress tolerance. Hence, the degree of the synergistic growth reduction effect of combined biotic- and high warming stress does not depend on the tolerance to high warming stress. Biotic stress tolerance is thus driven by other mechanisms than intrinsic growth rate and abiotic stress tolerance. We found that abiotic stress negatively affects the interactions between fungi, with likely important consequences for ecosystem functions [132], such as carbon sequestration. These results are opposite to the stress-gradient hypothesis (SGH) commonly observed in plant ecology, where interactions shift from more negative or competitive under control conditions to more positive or facilitative under stress conditions. However, also in plant ecology, this hypothesis lacks substantial evidence and is more driven by intuition, as the effects of abiotic stressors on plant species interactions depend on the type of the stressor and species variability [146], [149]. In fungal ecology, studies mainly focused on the identification of interaction types and quantified changes in dominance under different types of stress. For example, warming has been shown to reverse the outcomes of specific competitive interactions, with different fungi dominating under different scenarios [131]. Such studies have documented the potential ways in which fungi can interact and quantified their outcome, but lack a quantitative measure of species interactions. Hence, in order to be able to judge whether our observation of a reversed SGH can be extrapolated to fungal ecology in general, more

## General Discussion

experiments have to be conducted that explicitly quantify taxon specific interactions under abiotic stress.

In contrast to what could be expected based on the trade-offs of the plant ecology CSR framework, we thus did not observe a negative (nor positive) relationship of intrinsic growth rate nor abiotic stress tolerance with biotic stress tolerance within our heathland soil fungal community. This might be caused by the dominance of facilitative rather than competitive interactions among our heathland soil fungi, especially under benign conditions, suggesting that our quantified biotic stress tolerance is not an explicit measure of competitive ability under the CSR framework. Additionally to our observation in Chapter 3 of the lack of predictability of abiotic stress tolerance based on several functional traits, the CSR trait-based framework does not match the life-history strategies of these heathland soil fungi. Hence, this further indicates that for the desired shift in fungal ecology to likewise trait-based approaches [77], [117]–[120], more empirical research is needed to validate such frameworks. Malik *et al.* [170] recently stated that C-S-R strategies indeed do not necessarily map clearly on to microbial systems, and therefore proposed a revised life history theory for microbes, thereby promoting the use of omics datasets on genes, transcripts, proteins and metabolites to quantify the traits that define their redefined Y-A-S strategies. We evaluated the growth of a focal fungus under competition with a more abundant resident. This represents a kind of ‘worst-case’ scenario. When it can grow under these conditions, its long-term coexistence is more likely. The importance of the initial composition of the fungal community for further succession, through priority effects [171], [172], has been illustrated in heathland soils [134]. Our results thus suggest that climate change could potentially affect fungal community succession and consequently carbon sequestration in unpredictable ways by differentially promoting or inhibiting growth of interacting fungal taxa. Identification of the mechanistic drivers of our observed non-random effects of abiotic and biotic stress on growth, other than abiotic stress tolerance and intrinsic growth rate, allows for a better understanding of how climate change affects soil fungal community dynamics.

## Potential consequences of the observed changes in fungal functioning for carbon sequestration

Because of the observed large variation among heathland soil fungal taxa in abiotic stress tolerance, biotic stress tolerance and their potential contribution to carbon sequestration (enzymatic capabilities as a proxy), we can thus expect changing community dynamics leading to changes in carbon sequestration. However, we did not find a significant relationship between abiotic stress tolerance and enzymatic capabilities, as species that show a high production of (per-)oxidative enzymes, can be considered potentially important for the decomposition of recalcitrant organic matter and thus the acceleration of the carbon cycle. This shows that temperature and water stress might not selectively affect taxa that are either good or poor for sequestration. Although, our *Saccharicola bicolor* taxon, which was highly sensitive to water stress, also showed the highest enzymatic activities, especially (per-)oxidases. Additionally, some of the most abiotic stress tolerant species, both towards warming and drought, *Umbelopsis autotrophica* and *Rhizomucor sp.*, are fast growing ruderal-type species that were shown to be completely unable to breakdown complex organic matter. This indicates that climate change could increase carbon sequestration by negatively affecting mostly taxa that are enzymatically diverse and powerful. We might not have identified this potential relationship because the enzymatic capabilities did not vary enough among taxa. Also, the unexpected lack of a positive relationship between abiotic stress tolerance and melanin content indicates that climate change would not select for more melanized taxa within the community, and thus not increase carbon sequestration through an increased input of recalcitrant organic matter via highly melanized fungal necromass. The consistent reductions in growth rate induced by another species under climate change might reduce overall activity and thus increase carbon sequestration. Additionally, despite the relationship being non-significant, the growth of taxa that are best able to withstand high temperature increases are less negatively affected by biotic stress, thereby giving them an additional advantage. However, we don't know how these two-species interactions propagate to multispecies fungal communities [135].

## **Perspectives in order to increase the predictability of the effects of climate change on heathland soil community functioning and carbon sequestration**

For a more robust, less speculative conclusion on how climate change affects heathland soil carbon sequestration through changes in fungal community dynamics, more experiments are needed that explicitly quantify how the observed biotic and abiotic stress-induced changes in fungal growth rates mechanistically translate into changes in carbon sequestration. Thereby, a key focus has to be the assessment of the relevance of the findings from these simple artificial experiments to the real world. Therefore, there is a need to validate the observed results in more realistic set-ups such as soil microcosms. Additionally, it is important to more elaborately assess fitness of fungi. However, fitness is something that is difficult to define and measure. Growth has been argued to be a good indicator of activity for fungi, as mycelial extension rates directly regulate their capacity to forage for and decompose organic matter [173]. But, quantifying growth (as biomass or surface area) should be complemented with responses at other levels, such as genomics, proteomics and metabolomics. Also, the importance of spatial scale is important to consider. For example, individual-based models which can simulate interactions between functionally different microbes in a spatially structured micro-scale environment, illustrated that so called 'cheaters', microbes that exploit resources without own investments, reduce decomposition rates [174], [175]. Furthermore, there is increasing evidence that spatial soil ecology can yield new insights with regard to understanding the factors that maintain and regulate soil biodiversity, as well as to how the spatial distributions of soil organisms influence both plant growth, competition [176] and plant community structure [177]. In conclusion, we advocate for the following four practical steps.

First, we advocate for additional taxon-specific screening experiments to unravel which traits, for example production of different osmolytes, shape tolerance to abiotic stress of these heathland soil fungi. Thereby, it is important that traits values are assessed during the exposure to abiotic stress, or at least in similar

set-ups. Also, volatile production during interactions should be assessed in order to identify the observed biotic stress responses. A second step is to expose fungi to warming and drought in a more realistic set-up than artificial petri-dishes, such as soil microcosms. In order to link community- with carbon dynamics, soil carbon content (or a proxy) can be measured in such soil microcosms, which thus allows for a more explicit comparison with *in situ* observed conditions. This is an important step as interactions in a natural soil environment are more complex than in two-dimensional *in vitro* experiments [178], whereby the outcome of both experimental approaches might therefore deviate from each other. Tiunov and Scheu [179] for example showed that species richness of fungi similarly affect decomposition in two experimental set-ups of varying complexity, but with differences in strength and underlying mechanisms of the observed changes. In small (10g) soil microcosms experiments, we measured CO<sub>2</sub> production through GCMS-headspace as a proxy for growth rate and found that for example *Humicolopsis cephalosporioides* as a monoculture showed similar responses to temperature and water stress as in the petri-dish experiment, thereby indicating that our results are independent of the complexity of the experimental set-up. However, for some species, CO<sub>2</sub> measurements varied considerably among replicates and were therefore unreliable, showing the sensitivity and complexity associated with such microcosm systems. In such a more complex microcosm set-up, we could also expect facilitative interactions among fungi, as fungal species that breaks down recalcitrant carbohydrates such as cellulose into simpler forms, enable subsequent colonization by ruderal fungi [157].

A third step would be to test whether the abiotic- and biotic stress-induced growth changes and associated changes in carbon cycling observed under monocultures and pairwise interactions in more complex set-ups propagate to multi-species fungal communities. But to be able to track abundances in these pairwise and multi-species microcosm experiments, we need a (molecular) approach to differentiate between taxa. One possibility we investigated was the design of taxon-specific primers that bind at unique parts of the ITS sequence. Despite promising results, this approach however needs to be further optimized in order to be a reliable method. A more direct and accurate, but also more



## General Discussion

expensive method would be fungal ITS amplicon sequencing. Within this third step, complexity can be further increased by simulating grazing stress by adding for example a collembolan predator. The effect of grazing stress on fungal interactions and community composition has been intensively studied by Crowther *et al.*, who showed that Collembola selectively graze on specific fungal taxa, thereby changing the outcome of fungal interactions and thus exert non-random effects on fungal community composition [80], [131], [180]. Additionally, adding a grazer allows us to investigate differences in abiotic stress tolerances among trophic levels, which is of major importance to predict the effects of climate change on soil carbon sequestration (Chapter 1). Also, it is relevant to consider other environmental changes, such as for example nitrogen deposition, as Matulich and Martiny [181] for example showed that nitrogen availability can have a stronger effect on fungal community composition and respiration rates than changing moisture and temperature. The community and carbon dynamics in these complex experiments can be modeled using approaches of varying complexity, whereby we advocate the use of spatially explicit approaches.

Fourth and finally, insights from these predictive microcosm experiments can be validated in a large ecotron set-up, where various climate change- and carbon related parameters can be precisely measured and controlled. Using such a comprehensive, complex and realistic ecotron approach increases predictability of how climate change affects important ecosystem functions [182]. These perspectives would eventually allow scientists to make robust predictive conclusions on how climate change affects heathland soil carbon sequestration. This will in turn help policymakers to refine and adjust their goals to achieve realistic climate adaptation measures linked to the Paris Agreement, providing information for managers towards effective habitat restoration and sustainable management practices.

## Conclusions

The importance of species interactions and abiotic stress tolerances towards the effects of environmental change on ecosystem functions has been extensively empirically demonstrated, whereby theoretical models have been proposed to predict the context dependence of the relationships between biodiversity and ecosystem functioning [183]. This thesis provides important insights regarding the effects of two main environmental stressors on the functioning of heathland soil fungi. We found the heathland soil fungal community to be relatively well adapted to warming and drought stress, but with a large variability in tolerances among taxa. Additionally, climate change generally reduces fungal growth rates compared to benign conditions due to competition with another taxon. The degree of this growth inhibition however also varies among taxa. These results indicate that climate change potentially induces shifts in fungal community composition and succession in unpredictable ways by differentially promoting or inhibiting growth of fungal taxa. Several perspectives would validate and further complement the gathered knowledge, by addressing how the observed changes in fungal growth rates propagate into more complex set-ups and more complex communities and eventually translate into changes in soil carbon sequestration [184], [185], a crucial ecosystem service [22]. Thereby, scientific research can combat the environmental change that society has caused.



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# **Appendix**



## **Links between heathland fungal biomass mineralization, melanization and hydrophobicity**

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### **Abstract**

Comprehending the decomposition process is crucial for our understanding of the mechanisms of C sequestration in soils. The decomposition of plant biomass has been extensively studied. It revealed that extrinsic biomass properties, that restrict its access to decomposers, influence more the decomposition than intrinsic ones, that are only related to its chemical structure. Fungal biomass has been much less investigated in this respect, even though it contributes to a large extent to soil organic matter, and is characterized by specific biochemical properties. In this study, we investigated to which extent the decomposition of heathland fungal biomass was effected by its hydrophobicity (extrinsic property, governing access to hydrolytic enzymes from decomposers) and melanin content (intrinsic property). We hypothesized that, as for plant biomass, hydrophobicity would have a higher impact on decomposition than melanin content. Mineralization was determined as mineralization of Soil Organic Carbon (SOC) into CO<sub>2</sub> by headspace-GC/MS after inoculation by a heathland soil microbial community. Results show that decomposition was not affected by hydrophobicity, but was negatively correlated with melanin content. We argue that it may indicate that either melanin content is both an intrinsic and extrinsic property, or that some soil decomposers evolved the ability to use surfactants to gain access to hydrophobic biomass. In the latter case, biomass hydrophobicity should not be considered any more as a crucial extrinsic factor. We also explored the ecology of decomposition, melanin content and hydrophobicity among heathland soil fungal guilds. Ascomycete black yeasts had the highest melanin content, and hyaline Basidiomycete yeasts the lowest. Hydrophobicity was an all-or-nothing trait, with most isolates being hydrophobic.

## Introduction

Every year, the estimated increase of the atmospheric CO<sub>2</sub> pool is about  $3.3 \cdot 10^9$  Tons, to a large extent due to fossil fuel burning and land-use change [6]. Observations and estimations at the global scale indicate that terrestrial ecosystems actually affect this pool in a variable manner, depending on the years, going from a -0.9 sink to a  $+0.5 \cdot 10^9$  Tons/year source [5]. The outcome depends on the balance between C input rate through Net Primary Productivity (NPP), and C output rate by decomposition through heterotrophic respiration of the dead biomass into CO<sub>2</sub>. Therefore, in the last 20 years, many investigations were attempting to unravel which factors are regulating NPP and decomposition in terrestrial ecosystems, driving soil biologists to focus on a better understanding of the decomposition process, and, in particular, why is it slower in some ecosystems than others. A recently emerging view is that Soil Organic Carbon (SOC) decomposition is, at equivalent environmental conditions, influenced by its physico-chemical accessibility, and to a lesser extent by its chemical composition *per se* [186]. Indeed, compounds initially thought to be recalcitrant (such as lignin) were shown to have a faster turnover than expected, while the opposite was found for some small, more labile compounds [186]. Consequently, decomposition of SOC should be primarily retarded by conditions restricting its access, and only secondarily by its chemical structure.

The SOC originates from plant and root litter, root exudates, and microbial biomass. There is increasing evidence that microbes do contribute to a major part of SOC [186]–[188]. Indeed, fungi produce large amounts of biomass in soils, at the scale of 50 to 1000 kg/Ha (Cairney, 2012; Ekblad et al., 2013; Rillig, 2004) for mycorrhizal fungi, and 20-80 mg/kg of soil (Klein, McLendon, Paschke, & Redente, 1995) or 1000 kg/Ha for saprophytes (Watkinson et al., 2006). Despite its abundance in soils, however, the fungal biomass decomposition has received much less attention than plant litter, from which it differs by both the nature of structural molecules and physico-chemical accessibility. This is especially the case in heathland ecosystems, where the well-developed soil organic layer has been mostly assumed to be of plant origin, but where fungal biomass is also high [189], and is expected to better resist

## Appendix

decomposition [39]. This biomass can contain a fraction of structural compounds known to decompose slowly in soils. The most widespread of these molecules are melanins, which have a polyphenolic structure. Many fungi synthesize melanins to make their biomass resistant to a range of chemical or physical stresses [61]. Melanized fungi are frequent in the heathland ecosystem [39]. Consequently, at optimal environmental conditions, melanin content is often negatively correlated with the rate of decomposition. This has already been observed among fungi associated with forest trees [190]. Melanin content may therefore be considered an intrinsic property of the fungal biomass: it is a chemical property that does not affect the influence of environmental factors on decomposition [186].

However, some extrinsic properties of the biomass, which define how it interacts with the environment, do govern its stability in soils as well, and this to a much larger extent than chemical structure *per se* [186]. Solubility, for example, is one of the most critical factors limiting decomposition [191]. Indeed, most of the decomposition process relies on hydrolytic enzymes, or on enzymatic reaction steps requiring the substrate to be solubilized. Hence, the higher the hydrophobicity of fungal biomass, the slower should be its decomposition rate. However, this hypothesis has not been verified. The extent to which hydrophobicity influences fungal biomass decomposition has not been compared either with intrinsic properties such as melanin content.

Our aims were therefore twofold. First, we wanted to investigate how two key properties, one intrinsic (based on molecular structure: melanin content), and the other extrinsic (based on how the biomass interacts with its environment: hydrophobicity), were influencing the decomposition rate of dead fungal biomass. Our hypothesis was that these two properties were both significantly and negatively correlated with decomposition, but that hydrophobicity had more influence than melanin content, because it was restricting the access of decomposers to fungal necromass. We choose to test this hypothesis using fungal strains isolated from a dry heathland soil, where fungal biomass decomposition is poorly characterized while it is likely to be a major contributor to SOC. Second, we wanted to explore further how the

properties of fungal biomass varied between different fungal species and functional groups within the same ecosystem; whereby we hypothesized that these properties significantly differ between functional groups.

## Material & Methods

### *Sampling site*

This study was conducted in a dry heathland in the Nationaal Park Hoge Kempen (Belgium, 50°59'0.57"N 5°37'42.9"E). The area has a temperate climate, with an annual mean precipitation of 774 mm and a mean air temperature of 9.8°C. The dominant soil types are albic podzols and brunic-dystric arenosols (<https://dov.vlaanderen.be/dovweb/html/index.html>). In autumn 2016, a sampling plot of 50 by 60 meters was established in a dry heathland-dominated area of uniform vegetation and flat topography (50°59'01.9"N 5°37'39.8"E). The vegetation was six to seven years old (30-50cm height) and consisted of about 80 % *Calluna vulgaris*, 15 % *Molinia caerulea*, and 5 % bare soil, with lichens and mosses present under the canopy.

### *Fungal species isolation*

We tried to isolate as many species as possible from the heathland soil. For this purpose, we used a wide range of isolation methods (see SI). In total, 207 strains were isolated using all these procedures.

### *Fungal species identification*

In order to determine melanin content, hydrophobicity and mineralization rate of our isolated soil fungi, we needed to scale down the number of screened isolates to a manageable extent. Hence, we classified the 207 isolates into 26 different groups based on their morphological characteristics (growth rate, color, sporulation, growth pattern). One isolate of each of the 26 morphological groups was selected for this study. We identified the species by sequencing the isolate's ITS region. For this purpose, we collected a plug of actively growing mycelium (5mm diameter, 5mm deep), and inoculated it either into a 12-well plate containing 2ml of liquid Czapek-Dox medium, or a 250ml flask containing 100ml

## Appendix

of liquid Czapek-Dox medium, and incubated for three days (fast-growing species) to six weeks (slow-growing). The mycelial balls formed were then ground in liquid nitrogen using a mortar and a pestle, and DNA was extracted on this mycelial powder with the MoBio Powersoil DNA isolation kit. The ITS region was amplified using the ITS1f-ITS4 primers [192]. The PCR reactions were performed in a C1000 Touch Thermal Cycler (BioRad) in a mix composed of 10mM of each primer, 2mM MgSO<sub>4</sub>, 0.2mM dNTP mix, and 1 unit of Invitrogen Platinum Taq DNA polymerase High Fidelity PCR enzyme (Invitrogen Life Technologies, <http://www.thermofisher.com>). The PCR reactions were done using the following parameters: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72 C for 1 min, with a final extension at 72°C for 10 min. Amplification success was checked in a 1 % agarose electrophoresis gel in 1 % TBE (Tris-Buffer-EDTA) stained with GelRed. When amplification was not successful, we diluted the DNA template 20 times in TE buffer and added 20µl of mM BSA solution to the DNA sample before amplification. Amplicons were then sent to Macrogen for Sanger sequencing. The sequences were trimmed at both their 3' and 5' ends based on the visual inspection of electropherograms (poorly resolved peaks were removed). The sequences were then blasted on the NCBI database ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE\\_TYPE=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch)). The isolate was assigned to the taxon that appeared among the top hits with the highest e-value. In case several taxa had the same top e-value, we assigned the isolate to the one with the longest match. Based on literature, each isolate was then assigned to a group of similar ecology: black yeasts, hyaline yeasts, saprotroph, mycorrhizal fungi or endophytes. Data on species assignment are summarized in Table 1.

### *Mycelial hydrophobicity*

To measure mycelial hydrophobicity, we designed a device consisting of a microscope slide covered by a thin uniform layer of Czapeck-Dox (CD) agar (45.34 g.l<sup>-1</sup> Czapek-Dox medium, 5 g.l<sup>-1</sup> Yeast extract), laying in a Petri dish filled with 20ml of water agar medium (to avoid desiccation of the thin CD layer). The device was prepared as follows. First, the microscope slide was

sterilized by dipping in 96% ethanol and flaming on the Bunsen burner; then 1ml of hot CD agar was poured onto its surface using a 1ml micropipette, let to gel for five minutes, and transferred to the sterile water agar plate. The 26 fungal isolates were grown for a week on CD agar plates. Then, an actively growing plug of mycelium (0.3mm in diameter) was transferred to the middle of the slide. We prepared four replicates of each isolate (hence 104 devices in total). Devices were then incubated at 23°C in the dark for three weeks, after which slides were covered with at least 1cm<sup>2</sup> of mycelium. The mycelial hydrophobicity was then assessed by measuring the contact angle of water droplets deposited at the mycelial surface. This was done via sessile drop shape analysis as performed by [193]. We slightly modified this protocol: six water droplets of 2 µl were pipetted from one edge of the slide to the other edge on both sides of the inoculation point (Figure S1). For six isolates (the two *Penicillium velutinum* and the four *Umbelopsis autotrophica* ones), we used 10 µl droplets instead, since smaller ones were repelled by the substrate when being pipetted and ended up falling from the mycelial surface. Analyses were carried out at The Institute for Materials Research (IMO-IMOMEC) of Hasselt University. Contact angles were calculated using ImageJ (<http://rsb.info.nih.gov/ij/>). Measurements of contact angles were obtained by using the Low Bond Axisymmetric Drop Shape Analysis Model (LB\_ADOSA) plug-in, developed by [194](<http://bigwww.epfl.ch/demo/dropanalysis/>).

#### *Melanin content*

Mycelial melanin content of the isolates was assessed using the protocol of Gadd & Griffiths (1980). One actively growing plug (3mm in diameter) of each isolate was placed in a new CD agar plate, covered by a cellophane sheet which was previously sterilized by autoclaving. The 104 Petri dishes (26 isolates times 4 replicates) were then incubated for four to five weeks, depending on the growth rate of each isolate, in order to obtain a sufficient amount of biomass to perform melanin extraction. After incubation, the mycelium was scraped off the cellophane surface with a sterile scalpel and homogenized in liquid nitrogen using a sterile mortar and pestle. The homogenized samples were transferred to 50 ml Falcon tubes, and kept at -72°C. Samples were freeze-dried overnight in a

## Appendix

lyophilisator, and transferred to glass tubes. A 5ml solution of absolute ethanol was added to each tube, followed by heating in heating blocks at 60°C for 3 h. Next, samples were vortexed before being transferred to 15 ml Falcon tubes, and subsequently centrifuged for 10 min at 500 G. Supernatant was discarded and samples were again freeze-dried overnight in a lyophilizer, after which 1 ml of distilled water was added to the dried pellets, gently vortexing them before transferring them back to glass tubes. Next, samples were resuspended in 1 ml 6 M HNO<sub>3</sub>, and then placed in heating blocks at 75°C for three hours. 5 ml of distilled water was added to each sample. After vortexing gently, the resulting solution was transferred back to 15 ml Falcon tubes. Samples were again centrifuged (10 min, 500G), and the supernatant was discarded. Pellets were transferred back to glass tubes as described before. The resulting suspension was heated at 75°C for 20 min in 5 ml of 0.5 M NaOH, and filtered through grade 1 Whatman filter paper (Sigma). Melanin content was quantified by comparing the optical density of samples at 470 nm, with a standard curve generated using serial dilutions (0-40 mg/l) of synthetic melanin (Sigma), dissolved in 1 M NaOH.

### *Mineralization of C in fungal biomass*

The mineralization of the fungal biomass was assessed by measuring CO<sub>2</sub> production by a soil microbial community growing on a nutrient solution containing mycelial biomass as the sole C source, in a similar way as in McDowell *et al.* (2006). As [197] showed that the mineralization rates of ectomycorrhizal fungal biomass also depend on N content, we used a nutrient solution, ensuring that mineralization would be only limited by C quality (this is also how [196] ran their experiment). Briefly, mycelial biomass was prepared as for the measurements of melanin content (growth in CD agar covered by a cellophane sheet and homogenization of the mycelium in liquid nitrogen, then storage of the biomass at -72°C). A soil microbial inoculum was obtained as follows: on January 24<sup>th</sup> 2017, eight topsoil samples (8cm diameter, 5cm deep) were taken, every 5 m along two transects parallel to the longest side of the plot (four cores per transect). Samples were acclimatized at room temperature for two weeks, due to collection in winter conditions. Afterwards, samples were sieved at 2mm,

mixed thoroughly and pooled. One gram of this pooled soil sample was added to a 15ml Falcon tube, and mixed with 10 ml of sterile distilled water. The mixture was centrifuged for five minutes at 2000 rpm, and the supernatant was collected and used as heathland microbial soil inoculum. The nutrient solution contained 1.2 mM KCl, 0.5 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{KNO}_3$ , 0.5 mM  $\text{NH}_4\text{Cl}$ , and 0.1 mM  $\text{K}_2\text{HPO}_4$ . A headspace vial was then filled with 5ml of distilled water, 50  $\mu\text{l}$  of the nutrient solution, 50  $\mu\text{l}$  of heathland microbial soil inoculum, and 7.5mg of homogenized, dry mycelial biomass, and sealed with an airtight cap. Negative controls were prepared by preparing three vials containing only distilled water, and three other containing distilled water, nutrient solution and soil inoculum, but no C source. After one week, the  $\text{CO}_2$  concentration in the vial gas phase was measured by headspace-GC/MS.

### *Statistics*

We tested if the mineralization rate could be explained by hydrophobicity or the melanin content of the mycelium as predictor variables using a linear model. The normality of each of the three variables was assessed using a Shapiro test (at a  $p=0.01$  threshold); variables were transformed when possible to fit a normal distribution. The mineralization rate followed a normal distribution, as well as the log-transformed melanin content. Hydrophobicity could not be coerced to a normal distribution, and was instead separated into three categories: hydrophilic (contact angle=0 degrees, 16 data points), moderately hydrophobic (contact angle between 37 and 42 degrees, 8 data points), and hydrophobic (contact angle between 134 and 145 degrees, 101 data points). Statistical analyses were performed using R [198].

## **Results**

### *Mycelial hydrophobicity*

In this experiment, we assessed the mycelial surface hydrophobicity of the 26 heathland soil fungal isolates using the sessile drop contact angle measurements. We expected that mycelial hydrophobicity would be, as most functional traits, either normally or inversely distributed. Results showed



## Appendix

trimodal values instead, with three types of surfaces of separate hydrophobicity properties (Figure 1). Most isolates had a hydrophobic surface (angle between 135° and 140°). Only 6 of the 26 were hydrophilic to some degree. Two *Trichosporon porosum* isolates had a contact angle of 35-40°. The three *Exophiala spp.* and one *Rhizoscyphus ericae* isolates were extremely hydrophilic, to the extent that a drop of water was immediately spread over the surface of the mycelium, leaving no angle to measure. Hence, we assigned to these measurements a value of 0°. The three *Exophiala spp.* strains showed a differentiated mycelial surface, mostly covered by a smooth, highly hydrophilic basis that immediately absorbed moisture, on top of which sparse hydrophobic patches could be found (Supplementary Figure 2).

### *Melanin content*

This experiment was designed to measure the melanin content of all the 26 heathland soil fungal isolates, using the method of Gadd & Griffiths (1980). Since all strains displayed a large range of colorations, from pure white to totally black (Table 1), we expected melanin content to vary in the same proportions. Results showed that indeed there was a wide, two orders of magnitude range of melanin contents among the isolates, ranging from 1 to 170mg/g DW. Black yeasts had the highest melanin content (from 58 to 170 mg/l, Figure 2). One strain of *R. ericae* and one of *U. autotrophica* also displayed high melanin contents (above 50mg/g), while all other strains had low values (below 20mg/g). The four *Trichoderma viride* strains were all especially low in melanin (all below 5mg/g). When melanin contents were plotted against hydrophobicity, strains very clearly subdivided into four categories: melanized and hydrophilic (n=16), hyaline and moderately hydrophobic (n=8), hyaline and hydrophobic (n=76) and melanized and hydrophobic (n=4) (Supplementary Figure 1). Most of the strains were therefore hyaline and hydrophobic. None of the hyaline strains were hydrophilic.

### *Mineralization of C in fungal biomass*

The aim of this measurement was to assess the rate at which the C in the biomass of each isolate was decomposed into CO<sub>2</sub> by a local soil microbial

community. As for melanin content and hydrophobicity, we expected that biomass mineralization rate would be normally or inversely distributed among soil fungal isolates. We found that all isolates underwent significant degradation within a week of inoculation (Figure 3), since all produced a CO<sub>2</sub> signal at least 300 times higher than the blank without C substrate (12.22 at least in the isolate samples against 0.04 for the blanks, too small to be visible on the Figure). The blanks were filled with ambient air, hence at least 400ppm CO<sub>2</sub> and 80% N<sub>2</sub>. The negative control (nutrient solution, inoculum but no C source) showed that the inoculum itself was not significant as a C source (peak ratio=0.04). Results showed high diversity, both within and among species and functional groups. The amplitude of the differences was much lower than for melanin contents, though, with a factor 2 only between the slowest and the fastest mineralizing isolates. The isolates with the lowest degradation rate were: *Penidiella* sp.\_100, *R. ericae*\_106, *Saccharicola bicolor*\_49, the two *P. velutinum*, as well as *U. autotrophica*\_101. In opposite, *Trichoderma viride*\_72, *Umbelopsis autotrophica*\_32, *Trichoderma viride*\_9, *Trichosporon porosum*\_17 and *Trichosporon porosum*\_15 had the highest mineralization rates. All functional groups displayed very similar levels of degradability in average, so isolate identity accounted for most of the variance in this variable.

#### *Relationship between melanin content, hydrophobicity and C mineralization rates*

The aim of this experiment was to test the hypothesis that fungal biomass mineralization rate depended more on its surface hydrophobicity than on melanin content. Results showed that melanin content predicted decomposition of the fungal biomass, but surface hydrophobicity did not (Table 2). Within each hydrophobicity category, there was no correlation between mineralization rate and contact angle values (data not shown). Considering the limited number of points, we could not test this relationship between functional groups. However, it was clear that despite high differences in melanin content and hydrophobicity, mineralization rates were similar between functional groups.

**Discussion**

We investigated to which extent the biomass of heathland soil fungi differed in mineralization rates, and if these rates were best explained by biomass hydrophobicity or melanin content. Results showed that mineralization rates were uninfluenced by hydrophobicity, but negatively correlated with melanin content. We also explored how these three parameters were related to fungal functional groups, and found that mineralization rates varied much more between isolates than between functional groups.

*Relationship between C mineralization rate, melanization and hydrophobicity*

Our hypothesis was that hydrophobicity is a parameter that defines how the fungal biomass interacts with the environment, by regulating access of hydrolytic enzymes to their substrate, and therefore should have a larger influence on its decomposition than its melanin content. In fact, our results did show the opposite. The more melanized the biomass, the slower its C was mineralized by a heathland soil microbial community, while hydrophobicity was not correlated with mineralization. Biomass melanization is a widespread character among fungi, and to understand the effects it can have on the mineralization rate, it may be useful to elaborate further on the physiological role of melanin. This polyphenolic compound deposits in the fungal cell walls where it complexes with proteins and carbohydrates [61]. Melanin bears many similarities in its structure with lignin or lignin building blocks; it is therefore itself a stable compound, that can be degraded only by fungal peroxidases produced by white-rot fungi (Butler & Day, 1998). As for lignin in plants, it can be considered an intrinsic property of fungal biomass. Our observations confirmed the hypothesis that melanization and decomposition rate were negatively correlated. However, this correlation was not always tight: several isolates were melanized but still decomposed fast (*Exophiala* sp.\_96, *Exophiala* sp.\_94), others hyaline and recalcitrant (*Saccharicola bicolor*\_49). We expected that this variability would be explained by the hydrophobicity of the biomass, another factor that commonly hampers decomposition of organic molecules in soil [199]. Our results show, however, that hydrophobicity did not explain the observed mineralization. Hence, in our experiment, substrate accessibility for

hydrolytic enzymes did not play a role in decomposition, while melanin content, an intrinsic property of SOC, significantly did. To explain such unexpected results, one could formulate two hypotheses: (1) That melanin is at the same time an intrinsic and extrinsic property of SOC, because it also regulates its accessibility. Indeed, melanin molecules have the property to bind large amounts of water (only 10mg of melanin "granules" -bodies of agglomerated pigments- able to bind 1ml of water (Butler & Day, 1998)). By doing so, they cause the cell wall to swell to a significant extent (Fernandez & Koide, 2013), especially considering that melanins can contribute up to 25% of the fungal dry biomass (Fernandez & Koide, 2014). A thicker cell wall takes more time to be processed by cell-wall degrading enzymes (Kersten & Cullen, 2007), which retards all biomass decomposition. Moreover, in the same way as other polyphenols such as tannins, melanins can bind to proteins. This includes cell-wall degrading enzymes, where melanin binding potentially inhibits their activity (Ray & Desai, 1984). Hence, melanin content is both an intrinsic and extrinsic parameter of fungal biomass, and its relative influence on decomposition rates may consequently be high. (2) As for hydrophobicity, we cannot rule out either that some degrading organisms developed the ability to produce surfactants to improve their access to hydrophobic organic matter [200]. We did not verify surfactant production in our experiment, and do not know if this trait is widespread among the microflora in heathland soils. In such case, hydrophobicity should not be considered any more a crucial extrinsic factor for biomass decomposition in soils.

#### *Ecology of fungal hydrophobicity and melanization in the heathland ecosystem*

Mycelial melanin contents were in line with literature. Fernandez & Koide (2014) reported mycelial contents ranging from 39 to 248 mg/g, though this was measured on ectomycorrhizal fungi. In the same paper, the highest values were measured on isolates of the black ascomycete *Cenococcum geophilum*, and the lowest on hyaline basidiomycete isolates. Even though Ascomycetes were more dominant in our soil samples, we found a similar trend. The black yeasts had the highest melanin content, and hyaline yeasts (belonging to the Basidiomycetes) were at the other end of the spectrum.

## Appendix

We found that hydrophobicity was an all-or-nothing trait among our isolates, most of them being very hydrophobic, and a few being very hydrophilic. The dominance of the hydrophobicity trait among isolates was expected, since it is often associated with a better water retention strategy [201], and as stated above this is a crucial trait in dry heathland soils. However, in the literature mycelial hydrophobicity displayed more gradual figures than what we measured [193]. The large proportion of hydrophobic strains probably originates from the fact that the dry heathland environment selects for hydrophobic species. Indeed hydrophobicity may provide better water retention in case of drought, and to some extent better resistance to flooding [201], two common stresses in the well-drained sandy soil of dry heathlands under a rainy Atlantic climate [202]. This does not explain, however, the very few numbers of moderately hydrophobic strains. Mycelial age should not have been a confounding factor in our experiment [203], since it was considered in the experimental setup: water droplets were placed at increasing distance from the colony age, creating an age gradient. Our results demonstrated that age did not affect at all hydrophobicity measurements. However, since the slide was covered with only a thin layer of agar medium, mycelium growing atop had only access to a limited amount of nutrients; most of our strains may therefore have been well within their idiophase growth, which is known to favor aerial hyphae formation [203]. We would therefore turn moderately hydrophobic species into hydrophobic ones because of nutrient starvation during the experiment. In this case, however, it is not clear why fast-growing strains such as *T. porosum* did not show higher hydrophobicity, since they must have experienced nutrient starvation earlier than the slow-growing ones.

Finally, it was striking that most hydrophilic strains were very melanized. This seemed to be due to heterogeneity of mycelial surfaces, with hydrophobic patches surrounded by a very hydrophilic matrix. This latter substrate did not appear to be age-related, nor caused by any stress. It may be an artifact due to culture conditions, but also reveal its natural habitus in the soil. Such heterogeneity is sometimes observed in other fungal cultures [201]. It has been interpreted as a way for the fungus to balance between substrate exploitation (hydrophilic), nutrient translocation and stress resistance (hydrophobic). In any

case, this heterogeneity did not appear to affect mineralization rate of the isolates.

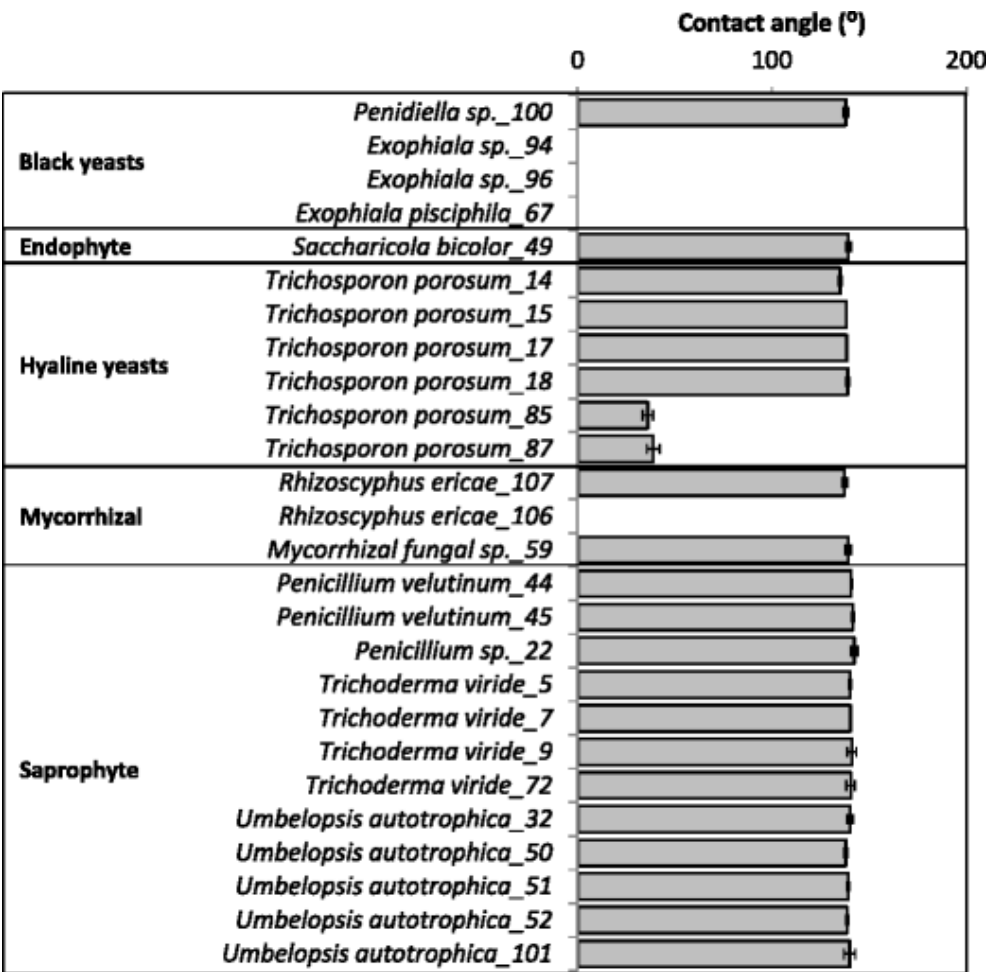
## Tables and figures

**Table 1:** Description of the isolates.

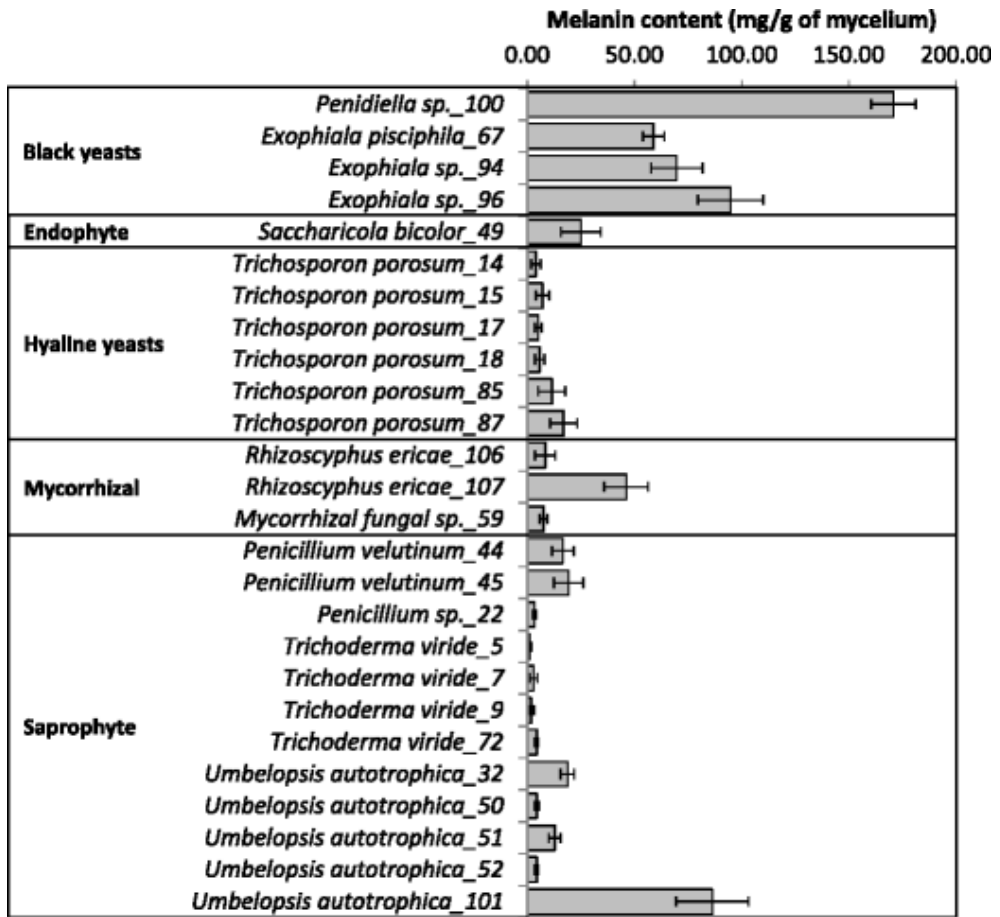
Strain	Species name	Functional group	Color	Growth rate	Taxonomic group	Isolation medium	Isolation method
100	<i>Penidiella</i> sp.	Black yeast		Slow	Ascomycota	MMN	Immersion tube (dilution plate)
94	<i>Exophiala equina</i>	Black yeast		Slow	Ascomycota	Pectin-agar	Immersion tube (dilution plate)
96	<i>Exophiala equina</i>	Black yeast		Slow	Ascomycota	Cellulose-agar	Dilution plate
67	<i>Exophiala pisciphila</i>	Black yeast		Slow	Ascomycota	Czapek-dox-agar	Dilution plate
49	<i>Saccharicola bicolor</i>	Endophyte		Average	Ascomycota	Water-agar	Soil plate
85	<i>Trichosporon porosum</i>	Hyaline yeast		Fast	Basidiomycota	Pectin-agar	Immersion tube (dilution plate)
87	<i>Trichosporon porosum</i>	Hyaline yeast		Fast	Basidiomycota	MMN-agar	Immersion tube (dilution plate)
14	<i>Trichosporon porosum</i>	Hyaline yeast		Fast	Basidiomycota	Pectin-agar	Soil plate
18	<i>Trichosporon porosum</i>	Hyaline yeast		Fast	Basidiomycota	Lignin-agar	Dilution plate
17	<i>Trichosporon porosum</i>	Hyaline yeast		Fast	Basidiomycota	Water-agar	Dilution plate
15	<i>Trichosporon porosum</i>	Hyaline yeast		Fast	Basidiomycota	Czapek-dox-agar	Dilution plate
107	<i>Hymenoscyphus ericae</i>	Mycorrhizal		Slow	Ascomycota	Cellulose-agar	Maceration
106	<i>Hymenoscyphus ericae</i>	Mycorrhizal		Slow	Ascomycota	MMN	Maceration
22	<i>Penicillium</i> sp.	Mold		Fast	Ascomycota	MMN	Immersion tube (dilution plate)
59	<i>Mycorrhizal fungal</i> sp.	Mycorrhizal		Slow	?	MMN	Immersion tube (dilution plate)
45	<i>Penicillium velutinum</i>	Saprophyte (mold)		Fast	Ascomycota	Czapek-dox-agar	Immersion tube (dilution plate)
44	<i>Penicillium velutinum</i>	Saprophyte (mold)		Fast	Ascomycota	Czapek-dox-agar	Dilution plate
5	<i>Trichoderma viride</i>	Saprophyte (mold)		Fast	Ascomycota	MMN	Dilution plate
7	<i>Trichoderma viride</i>	Saprophyte (mold)		Fast	Ascomycota	Cellulose-agar	Dilution plate
9	<i>Trichoderma viride</i>	Saprophyte (mold)		Fast	Ascomycota	MMN	Soil plate
72	<i>Trichoderma viride</i>	Saprophyte (mold)		Fast	Ascomycota	Soil	Soil plate
101	<i>Umbelopsis autotrophica</i>	Saprophyte (mold)		Fast	Zygomycota	Water-agar	Maceration
51	<i>Umbelopsis autotrophica</i>	Saprophyte (mold)		Fast	Zygomycota	Water-agar	Maceration
50	<i>Umbelopsis autotrophica</i>	Saprophyte (mold)		Fast	Zygomycota	Czapek-dox-agar	Dilution plate
52	<i>Umbelopsis autotrophica</i>	Saprophyte (mold)		Fast	Zygomycota	MMN	Dilution plate
32	<i>Umbelopsis autotrophica</i>	Saprophyte (mold)		Fast	Zygomycota	Soil	Dilution plate

**Table 2:** Results of the linear model describing fungal biomass decomposition depending on surface hydrophobicity and melanin content. Mineralization was expressed as CO<sub>2</sub> production by a heathland soil inoculum growing on the isolate biomass as the sole C source, surface hydrophobicity as contact angle of the isolate mycelium, and melanin content as the amount of melanin per gram of fungal biomass.

Variable	Standard error	t value	p value
Melanin content	– 0.04	0.01	2e– 4***
Contact angle (hydrophobic)	– 0.26	1.15	0.82
Contact angle (moderately hydrophobic)	– 2.31	1.7	0.18
Intercept	19.25	1.18	< 2e– 16***

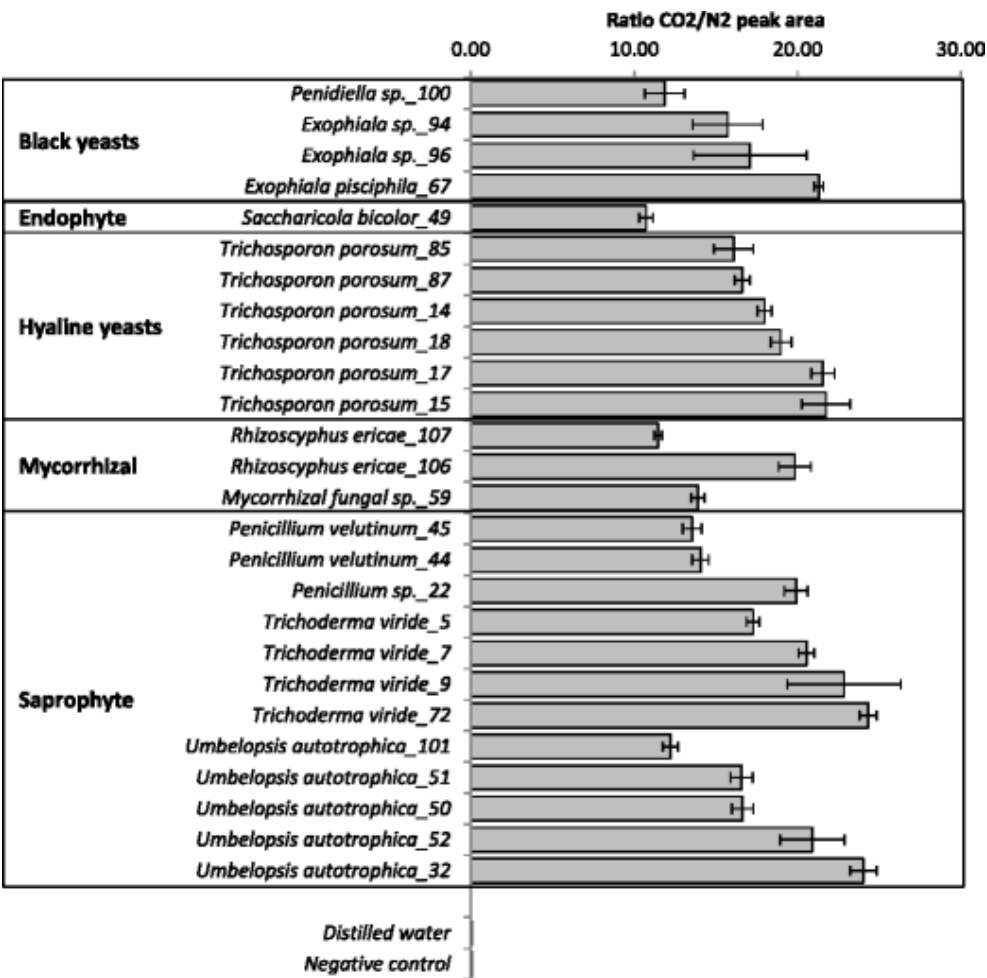


**Figure 1:** Hydrophobicity of the mycelial surface of the 26 heathland soil isolates (degrees). Hydrophobicity was measured as water droplet contact angle using the sessile drop analysis. Fungal mycelium was grown for one to four weeks on the surface of a sterile microscope slide covered by a thin layer of agar medium, placed on a water agar surface in a petri dish (to avoid desiccation). Results show the average and SD value of four slides per isolate. In each slide, six (exceptionally four for strain 101, where the mycelium area was too small to put six droplets) drops were measured. The higher the angle, the higher the hydrophobicity. Bars represent standard deviation between the four replicates (slides).



**Figure 2:** Melanin content of the mycelium of the 26 heathland soil isolates. Melanin content is expressed as mg melanin per gram of mycelium DW. All soil isolates were grown on Czapek-Dox agar medium in quadruplicate for four to five weeks, after which mycelium was collected, ground and freeze-dried, and used for melanin extraction. Bars represent standard deviation between the four replicates.





**Figure 3:** Carbon mineralization rate of the biomass of the 26 heathland soil isolates. Carbon mineralization rate was assessed by measuring CO<sub>2</sub> production after one week by a soil microbial community, using 7.5mg of dried fungal biomass as the only C source (and provided with the other nutrients). The biomass of each isolate has been quadruplicated. Bars represent standard deviation between the four replicates.

**Supplementary information**

*Methods of fungal strain isolation*

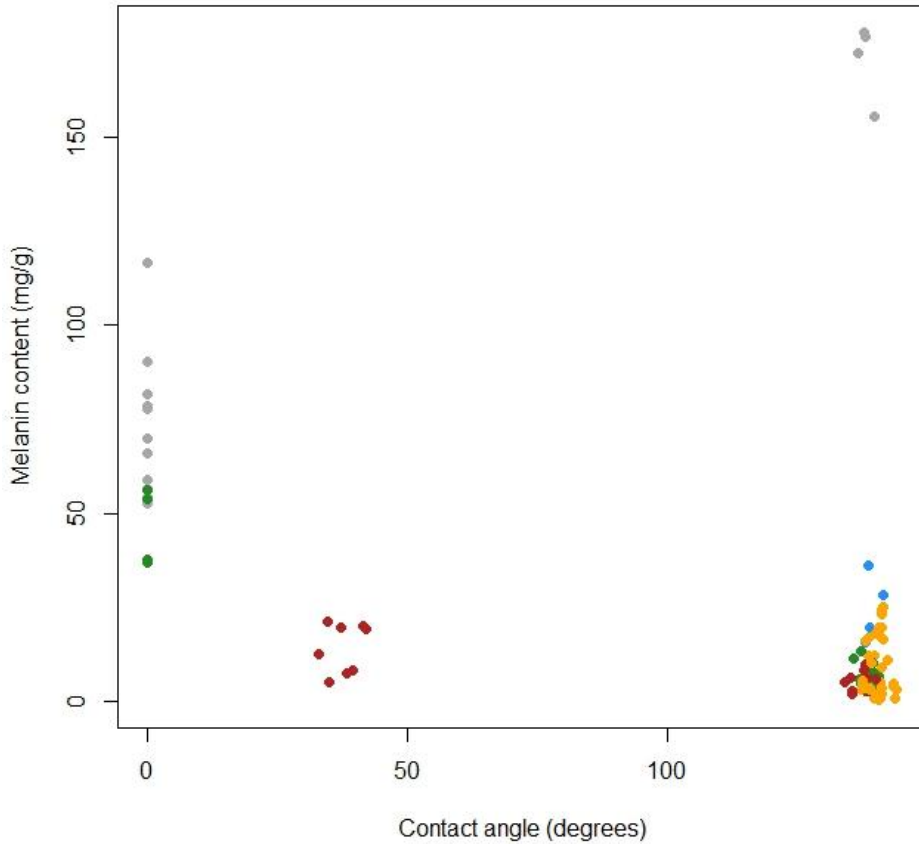
We tried to isolate as many species as possible from the heathland soil. For this purpose, we used a wide range of isolation methods:the soil plate (Warcup, 1950), the dilution plate, the root maceration, and Gochenaur’s (1964)

modification of the immersion tube (based on Chesters (1940; 1948)). The same soil samples were used for soil plate and dilution plate methods: eight soil samples (8cm diameter, 20cm deep) were taken, every 5 meters along two transects (four cores per transect). These transects were parallel to the longest side of the plot. The soil samples were immediately brought to the lab and sieved at 2mm, homogenized, and pooled altogether as one composite sample. For the soil plate method, 30mg of soil was added to the petri dish, and 20ml of agar medium was poured on top and gently swirled, in order to disperse soil particles both within and on the medium. The medium was poured just above stiffing temperature. For the dilution plate method, 1g of soil was diluted into 20ml of sterile distilled water, and we prepared 5 dilution series (10-1, 10-2, 10-3, 10-4, 10-5). A volume of 250 $\mu$ l of this suspension was spread with a sterile cotton swab on top of solid medium. The maceration method was used to isolate endophytes more specifically (Dark septate endophytes and mycorrhizae). For this purpose, we used two different approaches. First, based on (Pearson & Read, 1973): three *Calluna vulgaris* plants were collected and 20 roots were cut into pieces of 2cm, washed under running tap water for 2h and sterilized by serial washes in sterile water (20 times 5 minutes). We noticed however, that the cortical cells of the roots did not go in suspension, hence we inoculated the roots themselves on the growth medium (1 root per plate, 5 replicates per growth medium). Second, three other plants were unrooted and 50 tiny lateral roots (<1mm diameter) were washed under running tap water for 24h. Afterwards, they were surface sterilized for 1 min in 20% household bleach. Next, they were rinsed twice with sterile water. Using a potter, all root segments were together homogenized and partly macerated. A volume of 250 $\mu$ l of cell suspension was plated in each growth medium (5 replicates). Finally, we used immersion tube method as a way to isolate actively growing species (while the three other methods allow the growth of both actively growing and fungal spores). A volume of 1l of dry heathland top soil (top 20cm from the same plot) was autoclaved four times, and its sterility checked on growth medium. Then this soil was transferred to 15ml falcon tubes until they were half-filled, and 4 holes were made in different directions and different heights in the lower part of the tube (in sterile conditions). These so-called immersion tubes were brought to the field in a sterile packaging, and incubated in the soil of the sampling plot for

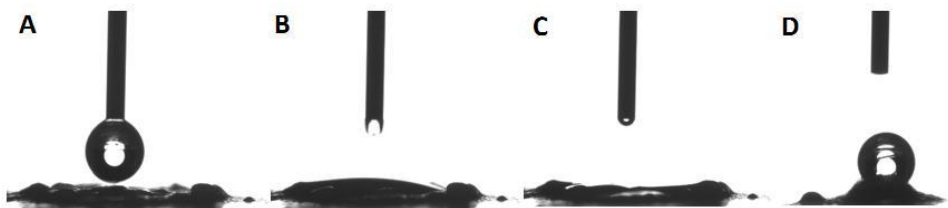
## Appendix

12 days (November 2015). Soil was taken from the tubes and processed through soil and dilution plate methods, as described above.

For all four methods, six different growth media were used: water agar (15g/l agar), MMN agar (2.5 g.l<sup>-1</sup> glucose, 500 mg.l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 200 mg.l<sup>-1</sup> NH<sub>4</sub>Cl, 150 mg.l<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 25 mg.l<sup>-1</sup> NaCl, 50 mg.l<sup>-1</sup> CaCl<sub>2</sub>, 12 mg.l<sup>-1</sup> FeCl<sub>3</sub>·6H<sub>2</sub>O, and 1 mg.l<sup>-1</sup> Thiamine-HCl; pH 4.0), Czapek-Dox with 0.5% yeast extract agar (45.34 g.l<sup>-1</sup> Czapek-Dox medium, 5 g.l<sup>-1</sup> Yeast extract), and three media based on Ingestad solution (Ingestad & Kähr, 1985) and a different C source: 0.4% cellulose agar, 0.4% pectin agar, and 0.4% lignin agar. The pH was adjusted to 5 in all media. The plates were then wrapped with parafilm, to avoid too much dehydration (especially for long periods of incubation), incubated in the dark at 23°C, and were kept for up to six months to allow late and slow growing species to germinate (Buerger et al., 2012). Once a strain started to grow, it was transferred to a Czapek Agar Broth medium and stored at 5°C.



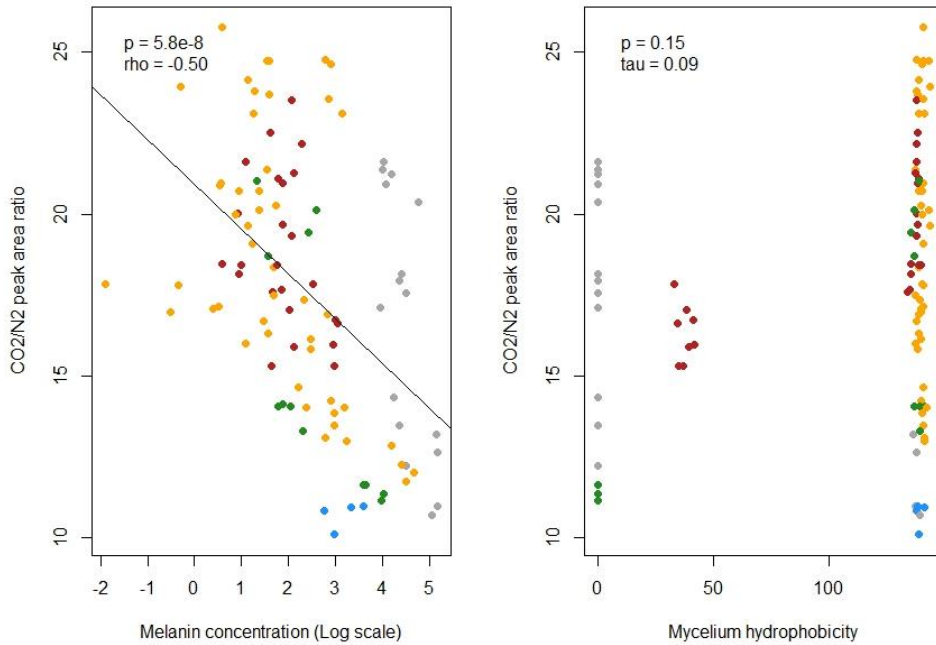
**Figure 4:** Plot of the melanin content in function of surface hydrophobicity. Each dot is one replicate, with four replicates per isolate. Dots are coloured per functional group. Orange dots=saprophytes, grey dots=black yeasts, red dots=hyaline yeasts, green dots=mycorrhiza, blue dots= endophytes.



**Figure 5:** Snapshots of drop placement on fungal slide culture of *Rhizoscyphus ericae*. A differentiated surface is apparent with a moist, very hydrophilic substrate covering most of

## Appendix

the mycelium (A-C). Sparse hydrophobic patches were found at the edges of the culture (D).



**Figure 6:** Correlations between carbon mineralization rate and melanin content in the mycelium (left), and carbon mineralization rate and mycelial surface hydrophobicity (right). The former correlation has been tested using Pearson correlation, the latter using Kendall. Orange dots=saprophytes, grey dots=black yeasts, red dots=hyaline yeasts, green dots=mycorrhiza, blue dots= endophytes.